Owing to the difficulties placed in the way of the printing trades by war conditions, *The Journal of General Physiology* desires to cooperate with the printer by eliminating unnecessary labor in the setting up of manuscripts. To accomplish this object, parts of the papers to be printed in different styles of type should be separated in the manuscript. For example, every experiment, table, or quotation of over five lines should begin on a new sheet. When the text is resumed, a fresh sheet should be started. Separate sheets should be used for the running head-lines, title, author, foot-notes, and bibliography. The name of the laboratory where the work was done together with the words, "(Received for publication,   , 1918)," should also be written on a separate sheet.

The entire manuscript should be copied with triple spacing.

In general the forms for headings, tables, references, etc., outlined in the "Suggestions for the preparation of manuscripts," issued by the Publication Department, should be followed. A copy of the "Suggestions" will be found on the back pages of the 25-Volume Index of *The Journal of Biological Chemistry.* We are glad to mail additional copies to contributors.

The *Journal* requests that, when possible, authors prepare their manuscripts in conformity with these suggestions.

ON THE DYNAMICS OF PHOTOSYNTHESIS.

By W. J. V. OSTERHOUT and A. R. C. HAAS.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

Although a great deal of attention has been paid to photosynthesis, nothing is known of the dynamics of the process. This aspect of the matter especially deserves investigation as furnishing a new point of attack upon this difficult problem.

We cannot analyze the dynamics of photosynthesis without first securing accurate data. A preliminary difficulty lies in the control of temperature; when leaves of land plants are exposed to sunlight, changes of temperature at once take place in the leaf and it is found that even under favorable conditions of control the temperature of the leaf may fluctuate as much as 10°C. in a half hour period. To avoid this difficulty, the writers have employed certain aquatic plants, which form thin layers or filaments, whose temperature can be regulated to a sufficient extent for the purposes of the investigation.

The fronds of the marine alga, *Ulva rigida* (sea lettuce), are so useful for this purpose that most of the experimental work was confined to them, although other material was used for comparison. These fronds consist of only two layers of cells and are so thin (about 0.078 mm.) that their temperature remains very close to that of the surrounding liquid. A further advantage of thin fronds is that gaseous exchange is extremely rapid.

The experiments on *Ulva* were carried on at the Marine Biological Laboratory at Woods Hole during the month of August when an abundant supply of excellent material was available.

To obtain data for the study of dynamics it is necessary to determine at frequent intervals how much photosynthesis has taken place. None of the available methods was satisfactory for our purpose. The method of counting bubbles is open to serious objections while the method of analyzing the gases in solution, as developed by Black-

1

man and Smith,[1] is not sufficiently accurate and convenient. The difficulty was solved by developing a method which depends on the fact that as the plants abstract carbon dioxide from the solution it becomes more alkaline.

In collaboration with Loeb[2] one of the authors had observed that certain marine algæ when exposed to sunlight cause the sea water to become more alkaline. Similar observations had been previously made by various observers[3] upon fresh water plants in solutions containing bicarbonates. If bicarbonates are absent, little or no effect is observed. The greater degree of alkalinity produced in the presence of bicarbonates is due to the fact that the plants abstract CO_2 from bicarbonates. Thus in sea water (which normally contains carbonates and bicarbonates) the alkalinity produced in this way may amount to more than $pH = 9$.

In the case of marine plants it is not necessary to add bicarbonates, since the sea water contains a sufficient amount. Such plants can therefore be studied in their natural environment, which is a distinct advantage over the methods hitherto employed, in which concentrations of CO_2 greatly in excess of the normal were maintained during the experiments.

In this connection it may be mentioned that some authors state that the concentration of free CO_2 is about the same in solutions containing carbonates and bicarbonates as in the air above and that plants in such solutions have no more CO_2 at their disposal than land plants. Aside from the fact that the amount of free CO_2 in sea water is not known, they seem to overlook the fact that when free CO_2 is abstracted from a solution of carbonates and bicarbonates it is at once partially replaced as the result of the dissociation of the carbonates and bicarbonates, so that the plant receives at once what otherwise must be more slowly supplied by diffusion. The carbonates and bicarbonates constitute a reservoir of CO_2 which may be depleted by photosynthesis during the day and filled up during the night by

[1] Blackman, F. F., and Smith, A. M., *Proc. Roy. Soc., Series B*, 1911, lxxxiii, 374.

[2] Loeb, J., The dynamics of living matter, New York, 1906, 98. Cf. Moore, B., Prideaux, E. B. R., and Herdman, G. A., *Proc. and Tr. Liverpool Biol. Soc.*, 1915, xxix, 233.

[3] Czapek, F., Biochemie der Pflanzen, Jena, 2te Aufl., 1913, i, 519.

diffusion from the air. The amount of CO_2 at the immediate disposal of the plant therefore depends largely on the amount of carbonates and bicarbonates present.

The usefulness of carbonates and bicarbonates in this connection is greatly increased by the fact that the plants are able to split them so as to extract from them much more CO_2 than can be removed by bubbling a stream of hydrogen through the solution.

In order to measure the degree of alkalinity produced by *Ulva*, a piece of the frond was placed in a tube of Pyrex glass[4] (about 12 mm. in diameter and about 5 cm. long) in such a manner that it completely covered the inside of the tube for the greater portion of its length. Fronds were chosen which were sufficiently stiff, so that their own elasticity caused them to remain pressed against the inner surface of the glass tube even when liquid was poured in and out or shaken back and forth in the tube.

The glass tube was sealed off at one end, while at the other it was furnished with a short piece of rubber tubing covered with paraffin.[5] The covering of paraffin was continuous and care was taken to renew it frequently.

After placing the frond in the tube, the latter was filled with sea water containing indicator[6] and the rubber tube was clamped shut. In some cases a small bubble of air was left in the tube to act as a stirrer; in other cases the tube was completely filled with sea water and the stirring was effected by a small piece of paraffin or by a glass bead covered with paraffin.

In order to determine the degree of alkalinity produced by photosynthesis two methods were used. In the first, the indicator was added to the sea water containing *Ulva* after a definite exposure to

[4] This glass was chosen because it does not give off measurable quantities of alkali during the period of the experiment.

[5] It is necessary to use paraffin which will not give off measurable quantities of acid during the time of the experiment. For this purpose paraffin of a high melting point is usually advantageous. Rubber should be used which gives off the minimum amount of acid; the rubber used in these experiments was repeatedly boiled before using.

[6] Ten drops of saturated alcoholic phenolphthalein was added to 1 liter of sea water. This makes the concentration of alcohol 0.0067 M and that of phenolphthalein 0.0001 M.

sunlight; in the second, the indicator was added to the sea water before the exposure began. In the latter case there was a possibility that the presence of the indicator might affect the amount of photosynthesis but it was found by control experiments that this was not the case with the concentrations employed in these experiments.

There is an advantage in adding the indicator at the start since this permits us to compare the times required to produce a given amount of change under different conditions. A comparison of the values thus obtained is more valuable than a comparison of the amounts of CO_2 abstracted during equal times, for the former procedure compares the reaction velocities accurately while the latter may not. In case the indicator is added at the end, instead of at the beginning, curves may be constructed (plotting CO_2 against time) from which the time required for equal changes in alkalinity may be obtained by interpolation.

In case any substance is added to the solution which changes its buffer value, due allowance must be made for this fact. An apparatus for determining the buffer action of added reagents has recently been described by one of us.[7]

It was found by preliminary experiments that the amount of CO_2 abstracted by the plant was an approximately linear function of the pH value (in the range here employed, between pH 8.1 and pH 8.3).

It was necessary to ascertain whether the degree of alkalinity produced was a reliable measure of the amount of photosynthesis. This was done by making simultaneous determinations of the degree of alkalinity and the amount of oxygen evolved (by a modification of Winkler's method recently described by us[8]). The results show that the amount of photosynthesis, as indicated by the evolution of oxygen, is approximately a linear function (in this range) of the change in the pH value of the sea water. This being so we can measure the amount of photosynthesis by determining the change in pH value regardless of any possible complications, such as excretion of alkali by the plant.

Since the plants produce CO_2 by respiration this must be taken into consideration. Experiments conducted under precisely the same conditions, except that light was excluded, showed that the respiration was

[7] Osterhout, W. J. V., *J. Biol. Chem.*, 1918, xxxv, 237.

[8] Osterhout, W. J. V., and Haas, A. R. C., *J. Biol. Chem.*, 1917, xxxii, 141.

practically constant. It is, therefore, easy to make a correction for it. It does not affect the form of the curve of photosynthesis found in the present investigation. That no acid other than carbonic is given off by the plant is shown by experiments in the dark in which the acidity produced was completely removed by a stream of hydrogen.

In order to ascertain how much photosynthesis had taken place after a definite time the pink color produced by the *Ulva* was matched against the colors of a series of Pyrex glass tubes[9] (of the same size) containing the same concentration of indicator in a series of buffer solutions of known alkalinity.[10] The matching was done under a "Daylight" lamp, which is invaluable for this purpose.

In this way the degree of alkalinity produced may be easily ascertained and since this corresponds to the amount of oxygen evolved, it gives us a direct measure of photosynthesis, provided we know the amount of CO_2 corresponding to the observed changes in alkalinity. This may be determined by the method referred to above.[7]

In order to carry out such investigations as the present one or to study the effects of temperature, light intensity, etc., it is not necessary to know the amount of CO_2 abstracted; it is sufficient to compare the time required to produce the same change in the color of the indicator under different conditions.

The experimental procedure was as follows: Young, vigorous plants of *Ulva* (deep green in color and not over 4 inches in diameter) were collected in the afternoon and placed in running sea water in the laboratory. In the evening they were covered with a dark screen so that the morning light could not reach them. On the following morning the plants were placed in a water bath at 27°C. and allowed to come to the temperature of the bath.

[9] These tubes were prepared and clamped shut in precisely the same manner as the tubes containing *Ulva*.

[10] Cf. Sörensen, S. P. L., *Biochem. Z.*, 1909, xxi, 131; *Ergebn. Physiol.*, 1912, xii, 393. Hoeber, R., Physikalische Chemie der Zelle und der Gewebe, Leipsic, 4th edition, 1914, 169. Bayliss, W. M., Principles of general physiology, New York, 1915, 203.

For the pH values needed in these investigations mixtures of 0.05 M borax and 0.2 M boric acid (to each liter of boric acid 2.925 gm. NaCl is added) are useful. The following table gives the pH values of a series of mixtures (Palitzsch, S., *Biochem. Z.*, 1915, lxx, 333; *Compt. rend. lab. Carlsberg*, 1916, xi, 199). Cf.

The experiment was started in the following manner. When everything (plants, sea water, and tubes) had come to the temperature of the bath, the plants were placed in the tubes, the rubber tubes were clamped shut, and the tubes were then placed in the bath so that they were about half an inch below the surface of the water, making an angle of about 20° with the surface, and exposed to direct sunlight.

Under these circumstances the alga receives abundant sunlight for photosynthesis. The amount of sunlight is affected by reflection from the surface of the water and the amount of reflection is influenced by the movement of the water due to stirring. But this is a fairly constant factor and of negligible importance. The effect of stirring can be obviated by placing a sheet of glass in contact with

McClendon, J. F., Gault, C. E., and Mulholland, S., *Carnegie Institution of Washington, Publication 251,* 1917, 21.

0.2 M boric acid.	0.05 M borax.	pH
cc.	cc.	
0	10	9.24
1.0	9.0	9.11
2.0	8.0	8.98
3.0	7.0	8.84
4.0	6.0	8.69
4.5	5.5	8.60
5.0	5.0	8.51
5.5	4.5	8.41
6.0	4.0	8.31
6.5	3.5	8.20
7.0	3.0	8.08
7.5	2.5	7.94
7.7	2.3	7.88
8.0	2.0	7.78
8.5	1.5	7.60
9.0	1.0	7.36
9.4	0.6	7.09
9.7	0.3	6.77

By plotting the cc. of borax as ordinates and the pH values as abscissæ a curve is obtained from which intermediate values can be obtained by graphic interpolation. From the pH values found in sea water 0.21 must be subtracted on account of the "salt error." In the present investigation these values were carefully checked by means of the hydrogen electrode.

the surface so as to eliminate ripples, or by using in place of the tub a tank with a vertical glass wall against which the tube is held in such a way that the light falls upon the tube after passing through the glass wall.

The temperature of the bath was kept constant within 1°C. There was no need of more accurate control of temperature under the conditions of the experiment. A thermometer was inserted into the tube in many of the experiments.[11] On exposure to sunlight the temperature of the tube rose and remained slightly above that of the bath, but under the conditions of the experiment the difference was almost constant. Since the tissues used were so extremely thin, it is safe to assume that the temperature of the tissues was practically the same as that of the liquid in the tube.

The tube containing *Ulva* was allowed to remain in sunlight, with occasional shaking to stir its contents, until a standard shade of pink was produced which matched that of a selected buffer solution (for purposes of matching a clear space was left in the tube above or below the frond). When the color of the tube was seen to be closely approaching that of the selected buffer solution, the tube was removed for an instant from the bath, shaken, and placed beside the selected buffer solution under the "Daylight" lamp. If the tint was not as deep as that of the buffer, the tube was returned to the bath. As soon as the desired tint was reached the time was noted, the sea water was emptied from the tube, and a fresh sample of sea water (containing indicator) was poured in. The tube was again exposed to sunlight and the time required to produce the same tint was noted. This procedure was repeated as often as necessary.

The experiments were carried out on cloudless days during the month of August. In case clouds interfered with the sunlight at any time during the course of the experiment the whole experiment was rejected. At first it was feared that the increase in the intensity of the sunlight during the morning and its decline during the afternoon might affect the results. In order to ascertain whether this was the case the experiments were started at various times during the

---

[11] The glass of the thermometer did not give off sufficient alkali to affect the results.

day. It was found that if the work was done between 9 a.m. and 4 p.m. the changes in the intensity of the sunlight might be neglected.

The results obtained are given in Table I, which represents the average of five experiments, and are illustrated in Fig. 1.

It is evident from Fig. 1 that the rate increases rapidly at first, then more and more slowly until it finally ceases to increase. From this point onward a steady rate is maintained.[12] The result is surprising, but in view of the fact that it has been confirmed by numerous experiments with *Ulva* as well as by experiments on *Enteromorpha*, *Spirogyra*, *Hydrodictyon*, *Potamogeton*, and other plants[13] it seems to be well established.

TABLE I.

Period.	Time required to produce standard alkalinity.	Total time exposed.	Amount of photosynthesis.	
			Observed.	Calculated.
	min.	*min.*		
1	35.7	35.7	1	0.92
2	25.9	61.6	2	2.07
3	23.3	84.9	3	3.18
4	21.7	106.6	4	4.23
5	20.4	127.0	5	5.23
6	20.3	147.3	6	6.22
7	20.5	167.8	7	7.22

Average of five experiments at $27° \pm 0.5°C$.

Two questions of great interest now present themselves. First, why does the rate increase at the start?[14] Second, why does it finally become stationary?

[12] This steady rate is not the same for each piece of frond but each piece is fairly constant in its rate, so that if the material is kept over night it will be found the next morning that different pieces may start out at different rates, but the steady rate which each piece finally attains is practically the same as the steady rate which the same piece had the day before. In fact it is possible to recognize the various pieces by this means.

[13] In experiments on fresh water algæ a small amount of sodium bicarbonate was added to the water.

[14] This acceleration is not due to the increase in the intensity of light as the sun gets higher for it was also observed when the experiments were started at noon.

. The suggestion which first offers itself is that photosynthesis belongs to the class of autocatalytic processes, in which the reaction is catalyzed by one of its own products. Such reactions begin slowly but as more of the catalyzing substance is produced the reaction goes on at an increasingly rapid rate until it begins to slow down as the reacting substances are used up. If these substances are constantly renewed, the reaction will not slow down but continue to go on more and more rapidly.
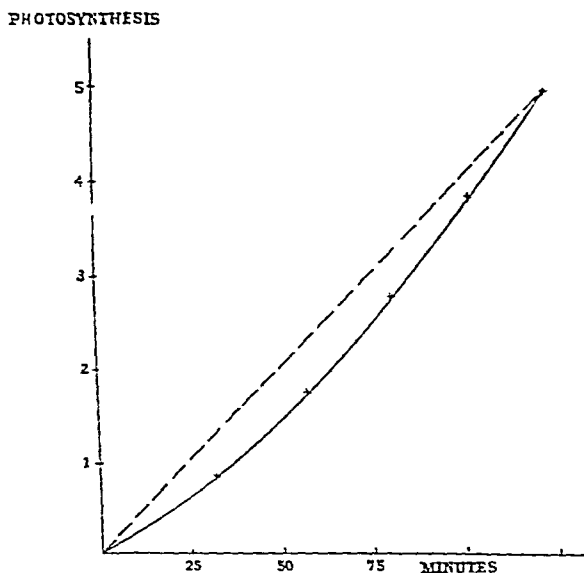
FIG. 1. Curve showing that where *Ulva* is exposed to light the speed of photosynthesis increases until a steady rate is attained. The dotted line expresses a uniform rate.

In our experiments on photosynthesis the reacting substances are constantly renewed.[15] The substances entering into the reaction are presumably carbon dioxide and water. The concentration of the water remains constant, while as soon as the concentration of the carbon dioxide has diminished by a very small amount it is brought back to the original point by the renewal of the sea water.

[15] When the sea water is not changed during the experiment the curve rises more rapidly at first, then bends over to the right as the supply of CO_2 is used up.

If photosynthesis were an autocatalytic reaction, the amount of catalyzer should increase in the manner indicated in Fig. 2 and, under these conditions, the process should continue to increase in speed as time goes on.   As a matter of fact it soon attains a steady rate.   This might be accounted for by supposing that the concentration of the catalyst cannot exceed a certain amount, being limited by its own solubility.   But in that case the rate would increase more and more rapidly up to a certain point and suddenly become stationary when the limit of solubility was reached, the curve of the catalyst being like that shown in Fig. 3.[16]   This is not the case.   The rate increases rapidly at first then more and more slowly until it finally becomes stationary.
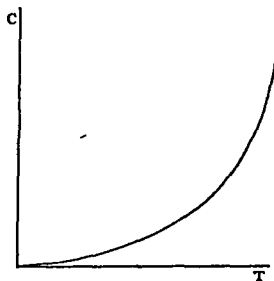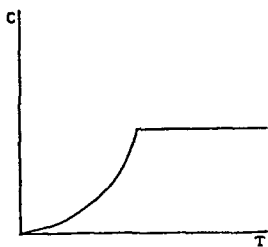
FIG. 2.   FIG. 3.

FIG. 2. Curve showing increase in the amount of the catalyst when the reaction is autocatalytic (C, catalyst; T, time).

FIG. 3. Curve showing behavior of the catalyst when its concentration is limited by such a factor as solubility (C, catalyst; T, time).

It might be supposed that the speed of the reaction is checked by the accumulation of the products of the reaction.   In that case, however, the rate would not become constant but would gradually diminish to zero.   Such influence of the products would be possible only in the case of a reversible reaction and we have no ground for believing that photosynthesis comes under this head.[17]

[16] This is because the catalyst from the moment of its production is in solution. It is not analogous to a solid going into solution, which dissolves more slowly as the limit of solubility is approached.

[17] While respiration is in a sense the opposite of photosynthesis the steps in the process are apparently quite different from those found in photosynthesis.

It might also be suggested that the rate becomes constant through the operation of a "limiting factor" such as lack of light, carbon dioxide, or of temperature.   But it is evident that the effect of such a factor would be fully felt at the very start of the reaction and that it could not cause a gradual falling off in the increase of speed.

This puts clearly before us a fundamental difficulty.   The fact that the rate increases most rapidly at first and then more slowly shows that photosynthesis is not an autocatalytic reaction in the usual sense of the word, for in such a reaction[18] the rate would increase slowly at first, then more and more rapidly as time goes on.   We must therefore conclude that photosynthesis belongs in a different category.
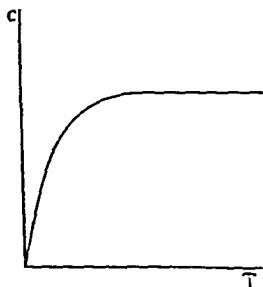
FIG. 4. Curve showing the behavior of the catalyst on the assumption that it is produced by the monomolecular reaction $A \rightarrow C$ (C, catalyst; T, time).

The key to the situation is furnished by the figures in the second column of Table I, which show that if the reaction is catalyzed by a substance, it must be produced more rapidly at first and then more and more slowly.   It is also evident that this substance must be limited in amount and that when its production ceases the rate of photosynthesis becomes constant.   We may assume that the rate of photosynthesis is proportional to the amount of the catalyst, which we will call C.   The figures suggest that this substance may be produced in the manner characteristic of a monomolecular reaction as shown in Fig. 4.   We may therefore assume that C is produced by a substance A, under the influence of sunlight, according to the monomolecular reaction: $A \rightarrow C$.

[18] I.e., under the conditions of the present experiment, where the reacting substances are kept approximately constant in composition.

We may now test this assumption by calculating the amount of photosynthesis which is to be expected after the lapse of a given time.

According to the ordinary equation for a monomolecular reaction,

$$C = A - Ae^{-KT}$$

in which T is time, e is the basis of natural logarithms, and K is the velocity constant of the reaction.

We may denote the amount of photosynthesis by P. If the rate of photosynthesis is directly proportional to the amount of C, we may, for convenience, put

$$\frac{dP}{dT} = C;$$

hence

$$\frac{dP}{dT} = A - Ae^{-KT}.$$

On integration this becomes

$$\frac{P}{A} = T - \frac{1}{K} + \frac{1}{K}e^{-KT}.$$

When the rate has become constant we find that a unit amount of photosynthesis is produced in 20.4 minutes (average of the last 3 periods in Table I), hence the rate of photosynthesis at that time is $1 \div 20.4 = 0.049$. This is by assumption equal to C when A is completely transformed into C and this is in turn equal to A at the beginning of the reaction. Hence A at the start = 0.049. We may substitute this value in the equation and find the value of K by trial. If we put $K = 0.049$ we get the values given in Table I. Better agreement with the observed values is obtained by taking lower values of K. This produces a gradual falling off in subsequent values, but it is possible that this might actually occur if the experiment could be continued for a sufficient length of time.

The agreement between the observed and the calculated values is very satisfactory except at the start. In this connection it may be pointed out that at the beginning of a reaction disturbances are to be expected.

It is therefore evident that the assumption justifies itself by giving an adequate quantitative explanation of the observed results. The

question then arises whether it is a natural one. It would seem very probable that the light produces a substance which accelerates the reaction and unless this substance is produced in unlimited amount there must come a time when the rate will become steady (or fall off). The assumption therefore seems to be reasonable.

It is attractive to form a hypothesis as to the nature of the catalyst. One might be tempted to suppose that it is chlorophyll but for the fact that some plants which are deep green may not photosynthesize as rapidly as those which possess less chlorophyll.[19] It is of course possible that the less active plants are deficient in some essential factor other than chlorophyll. On the other hand it may be necessary for chlorophyll to be transformed by the light from an inactive into an active form,[20] so that the rate of photosynthesis depends on the amount of "active chlorophyll" present. This would be analogous to the well known activation of enzymes by various means.

An equally satisfactory quantitative explanation is obtained if we suppose the amount of photosynthesis to correspond to the amount of a substance P, produced (under the influence of light) by the monomolecular reactions

$$S \rightarrow M \rightarrow P,$$
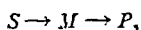
in which S represents a constant source (*i.e.*, a substance which does not appreciably diminish during the experiment).

Let us suppose that in the morning, before the frond is exposed to the light, S alone is present. On exposure to light the formation of M and P begins. The amount of M will then increase until it reaches a constant value (when its rate of formation is equal to its rate of decomposition) but the value of P will continually increase, since it does not undergo decomposition. When M has reached a constant value we find (putting K as the velocity constant of the reaction $M \rightarrow P$) that the amount of M decomposed in 1 minute (unit time) is KM; this is also the amount of P which is formed in 1 minute, and since the reaction $S \rightarrow M$ produces just enough of M to balance the loss of M (by transformation into P) the amount of M produced each minute

---

[19] Aquatic plants taken directly from ice-covered ponds in winter are found to possess but feeble photosynthetic power, though of a deep green color.

[20] Such activation of substances by light is well known in photochemistry.

is KM.   Hence if we start in the morning with S alone there will be produced each minute KM and all of this will be transformed into P except what is present at any moment as M.   Hence the amount of P produced in the time T is $KMT - M$.

When M has attained its constant value, we may, for convenience, put $M = 1$.   The rate of increase of P is then constant and we find from the table that it takes 20.4 minutes to produce one unit of photo-
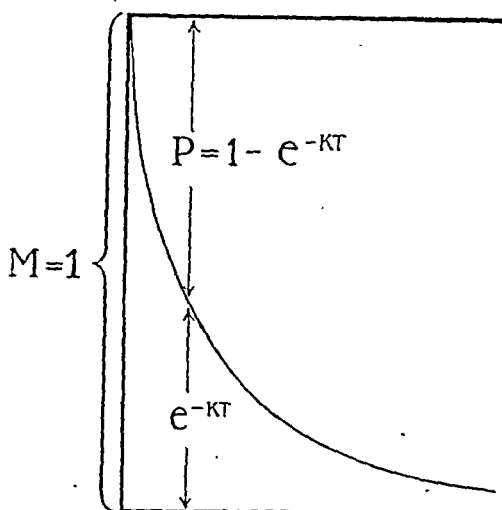
FIG. 5. Curve to illustrate that, as M decomposes in monomolecular fashion to form P, the amount of M left at any given time, T, is e^{-KT} and the amount of P is $1-e^{-KT}$.   The abscissæ represent time; the ordinates the amount of M. At the start of the reaction $M=1$.

synthesis; hence $KMT = 1$.   Substituting in this equation the values of M and T we have 20.4 $K = 1$, whence $K = 0.049$.

At the start of the reaction the value of M is 0; this gradually increases to 1 and remains constant.   During this period of increase the value of M may be calculated as follows: When M has reached its constant value ($M = 1$) let us suppose that the reaction $S \rightarrow M$ suddenly stops while $M \rightarrow P$ continues; we shall find that if T minutes have elapsed after this occurrence, the amount of M which has disappeared is $1 - e^{-KT}$ (see Fig. 5).   If the reaction $S \rightarrow M$ had not

stopped it would have produced enough of M so that (in spite of the fact that M is constantly decomposing) the amount of M remaining at the time, T, would be just enough to balance the loss, or $1 - e^{-KT}$. Hence if we start with nothing but S (the values of M and of P being 0) the amount of M present after the lapse of any given time T will be $1 - e^{-KT}$ and the amount of P will be

$$P = KT - (1 - e^{-KT}).$$

This is the same as the equation

$$\frac{P}{A} = T - \frac{1}{K} + \frac{1}{K} e^{-KT},$$

when in the latter we put $K = A$ as was done in making the calculations given in Table I. Hence when we substitute the value $K = 0.049$ in the equation $P = KT - (1 - e^{-KT})$, we obtain the values already given in Table I.

If the chlorophyll takes part in the reaction by decomposing or by combining (as some recent evidence indicates), we might suppose that S represents inactive chlorophyll, M active chlorophyll, and P a derived substance which combines with CO_2. At present it does not seem profitable to attempt a more extended discussion of this question. But it may be pointed out that (as one of us has recently emphasized)[21] consecutive reactions of the type here discussed are to be looked upon as the rule, rather than as the exception, in living matter.

It is evident that either of the theories developed above gives a quantitative explanation of the results. Both seem to be based on reasonable assumptions. Future investigation must decide which is more useful.

In any event, it is clear that much is to be learned concerning the dynamics of photosynthesis, and it is hoped that the considerations here set forth may be of value in this connection.

SUMMARY.

Minute amounts of photosynthesis in marine plants can be accurately measured by adding a little phenolphthalein to the sea water,

---

[21] Osterhout, J. Biol. Chem., 1917, xxxi, 585; xxxii, 23.

and observing changes in the color of the indicator. In the case of fresh water aquatics bicarbonates are added.

By this method it is found that *Ulva* which has been kept in the dark begins photosynthesis as soon as it is exposed to sunlight and that the rate steadily increases until a constant speed is attained.

This may be explained by assuming that sunlight decomposes a substance whose products either catalyze photosynthesis or enter directly into the reaction.

Quantitative theories are developed in order to account for the facts.

A METHOD OF STUDYING RESPIRATION.

By W. J. V. OSTERHOUT.

(*From the Laboratory of Plant Physiology, Harvard University, Cambridge.*)

In 1915 the writer suggested to Dr. Haas the desirability of experiments with indicators with a view to measuring the amount of CO_2 given off by organisms. The method finally developed by Dr. Haas[1] in the writer's laboratory consists in adding the indicator directly to the liquid in which the organism is placed. The indicator is usually present at the start, but in some instances it is not added until after the CO_2 has been produced.

The method is simple, accurate, and extremely satisfactory but it has obvious limitations. It is restricted to the use of aquatic organisms and furthermore it does not permit us to study the effect upon respiration of reagents which have a pronounced acid or alkaline reaction. There are also difficulties in using organisms or tissues which give off alkali or acid (other than CO_2). In addition, the use of small suspended organisms which color the solution must be avoided. A further disadvantage is that some reagents cause organisms to give off coloring matters which interfere with the results. The use of toxic indicators also presents difficulties.

These difficulties may be obviated by means of an apparatus designed by the writer. The purpose of the present article is to make clear the principle employed and to describe a simple form of the apparatus without mentioning other forms or discussing the modifications of mechanical details which are of importance for special purposes.

The apparatus shown in Fig. 1 consists of a bottle, A, in which the organisms are placed; the CO_2 which they exhale passes out of A through D into the indicator solution[2] in the Pyrex glass tube, B,[3]

[1] Haas, A. R. C., *Science*, 1916, xliv, 105.

[2] The tube which dips into the indicator solution should be of Pyrex glass. This glass is chosen because the amount of alkali given off is negligible.

[3] This tube should be about ¾ inch in diameter; the stopper should be covered with a paraffin which does not give off acid.

returning through the rubber syringe, F,[4] into A. A constant circulation[5] is kept up by the motion of K, each downward movement of which compresses the syringe, F, forcing a stream of bubbles[6] through the indicator solution in B; K is attached at one end to the hinge, L, and at the other to the connecting rod, G, which moves when H is made to revolve by means of a small motor. The syringe,
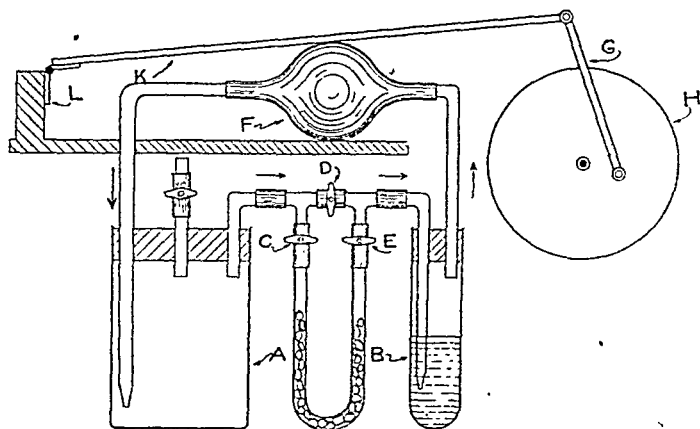
Fig. 1. Apparatus for the measurement of respiration and of photosynthesis. The organisms are placed in A; by compressing the syringe, F, the CO_2 is forced through the indicator solution in B, returning through F into A. The wheel, H, is caused to rotate by means of a motor; the resultant motion of K compresses F and keeps up a constant circulation of gas.

---

[4] This has valves at the outlet and inlet. In place of the syringe a tambour-bellows, pump, miniature fan, or other simple device may be employed.

[5] It is desirable to have the gas circulate at a constant rate. The rate may be controlled in a number of ways; it may be tested by observing the rate at which the color of the indicator changes, when a stream of air, free from CO_2, runs through it.

[6] If the stream of bubbles does not flow satisfactorily some of the solution may be removed so that the top of the tube is brought nearer to the surface. Inclining the tube, B, may assist in freeing the bubble as it issues from the tip of the tube. The compression of the syringe may also be increased and a syringe with stiffer walls may be used. If this does not produce the desired result the elasticity of the system must be increased by making the rubber connections longer, or by using softer rubber; or, better, a small elastic bulb may be introduced at any convenient place.

F, rests upon a board which is held in position by a support not shown in the drawing. The amount of compression of F can be varied either by shifting F or by altering the point at which G is attached to H.

A and B are provided with tightly fitting stoppers into which the tubes are fastened in air-tight fashion. All other connections should likewise be air-tight.

The carbon dioxide produced by the organism is absorbed with surprising rapidity by the indicator solution, provided a rapid circulation of air is maintained. This is evident from the fact that when known amounts of CO_2 are introduced into A the CO_2 is at once carried over into B and equilibrium is quickly established.

Ordinarily it is not important to know the absolute amount of CO_2 produced by the organism, since we are concerned only with comparative values. If for example we wish to compare the normal rate of respiration with the rate under the influence of ether, we proceed as follows: We place the organisms in A and close the clamp, D; opening C and E so that the air passes through the U-tube[7] (containing an absorbent of CO_2, such as lumps of NaOH)[8] into the indicator solution in B. The indicator solution may consist of distilled water (free from CO_2) to which enough NaOH has been added to make its pH value about 7.3; phenolsulfonephthalein is added, so that the solution becomes pale pinkish in color. As the gas passing into B is practically free from CO_2 little or no change will be produced by its circulation; it should, however, be allowed to circulate until the color of the indicator has become constant.[9] The clamps, C and E, are now closed and D is opened, allowing the CO_2 given off by the organisms to pass into B. The time required to produce a clearly marked change in the color of the indicator is noted and buffer solutions (contained in Pyrex glass tubes of the same size as B and having the same concentration of indicator) are selected which match the color of the indicator at the start and finish. The clamp D is now closed; C and E are opened, thus washing the CO_2 out of B until the indicator returns to

---

[7] In place of the U-tube a tower or potash bulb may be employed.

[8] When a volatile reagent, as ether, is added, the substance in the U-tube must be one which does not absorb the reagent.

[9] Unless the joints are tight CO_2 may leak in.

the original color. This is repeated until the normal time of respiration is established (unless it is fairly constant the experiment should be rejected).

. The reagent is now introduced into A (by means of the short tube) and the time is ascertained which is required to produce the same change in the color of the indicator. A comparison of this with the normal time gives the relative rate of respiration under the influence of the reagent.

The amount of CO_2 produced by the organisms is ascertained by comparing[10] the color of the indicator with the colors of a series of buffer solutions[11] having the same concentration of indicator and contained in Pyrex glass tubes of the same size as B. This determines the pH value of the solution. In order to find out how much CO_2 must be evolved to produce a given change in the color of the indicator we introduce known amounts of CO_2 into the apparatus[12] by means of a device which has recently been described.[13] The gas is made to circulate in the apparatus until the introduced CO_2 is thoroughly mixed with the air and with the indicator solution, so that equilibrium is established between the latter and the CO_2 in the apparatus. We know that this has occurred when continued circulation fails to produce any further change in the color of the indicator.

Instead of introducing gaseous CO_2 we may introduce a solution whose CO_2-content is known from its pH value.

The change in the color of the indicator produced by a given amount of CO_2 will vary according to the volume of air in the apparatus (the amount of indicator solution being constant). When determinations have been made for a number of volumes, intermediate values may be obtained by interpolation. As a rule there will be no changes in volume except those produced by variations in the volume of the organisms which are introduced into the apparatus.

Ordinarily the respiration of aquatic organisms may be studied by the method described by Haas[1] but such organisms as impart a color to the solution or give off acid (other than CO_2) or alkali may be

---

[10] This should be done, if possible, under a "Daylight" lamp.

[11] Cf. Osterhout, W. J. V., and Haas, A. R. C., *Science*, 1918, xlvii, 420.

[12] This can be done by means of the short tube in the stopper of A.

[13] Osterhout, W. J. V., *J. Biol. Chem.*, 1918, xxxv, 237.

studied by means of the apparatus here described. In this case A is partly filled[14] with liquid,[15] and due allowance must be made for this in calibrating the apparatus with respect to absolute amounts of CO_2 required to produce a given change of pH value. This calibration must be revised if any reagent is added which has a pronounced buffer action; this should also be done when A is filled with gas if any volatile substance with a pronounced buffer effect (or with an acid or alkaline reaction) is used.

In general it would seem to be desirable to have A large enough to contain a number of organisms, so as to reduce as much as possible the irregularities which may occur when small numbers are used. But if the amount of CO_2 given off is small, it may be necessary to reduce the size of A by substituting a U-tube for the bottle. The size of F may also be reduced by substituting a smaller bulb, or by using in place of a bulb a tambour provided with valves.

Whenever it becomes desirable to sweep out the exhaled CO_2 and to fill the apparatus with ordinary air, this can be easily and quickly accomplished, without disturbing the organism. It is only necessary to remove the U-tube and to close the screw clamp. D. (which is usually open) and open the screw clamps, C and E. (which are ordinarily closed) and then to start the motor and allow the gas to circulate, passing out of the apparatus at E. Air will then enter at C and will quickly wash out the excess of CO_2. When this is accomplished (as shown by the color of the indicator). C and E are closed and D is opened.

Ordinarily it will be found desirable to remove the excess of CO_2 at frequent intervals. In this case phenolsulfonephthalein will be found useful as an indicator.[1] If, however, CO_2 is allowed to accumulate, other indicators or mixtures of two or more indicators may be used to measure the lower pH values.

---

[14] Except in cases where the organisms respire normally when merely moistened and kept in a saturated atmosphere. Ordinarily the air will become partly saturated with the moisture taken up from the indicator solution. If this is not desirable, in the case of non-aquatic organisms, it can be obviated by adding something to the indicator solution to lower vapor tension or by drying the gas on its way from B to A.

[15] The tube by which the gas enters A is drawn to a point and brought down to the bottom of A to permit the gas to bubble through any liquid contained in A.

In case it is desired to withdraw a sample of gas in order to determine the amount of O_2 consumed, it is only necessary to intercalate a glass tube in the neighborhood of D, with stop-cocks at each end which can be closed when the tube is removed.

It is evident that this method is just as applicable to photosynthesis[4] as to respiration. In studying photosynthesis it may be desirable to introduce known amounts of CO_2 into the apparatus at the beginning of the experiment.

In conclusion it may be added that in carrying out such investigations it is desirable to compare the times required to produce equal amounts of CO_2 rather than to compare the amounts of CO_2 produced in equal times. The former method compares reaction velocities while the latter may not.

SUMMARY.

An apparatus is described which makes it possible to measure rapidly and accurately small amounts of CO_2 given off by organisms of all kinds. The apparatus can also be used to measure photosynthesis.

THE ANTAGONISM BETWEEN THYROID AND PARATHYROID GLANDS.

By EDUARD UHLENHUTH.

INTRODUCTION.

It is well known that the extirpation of the parathyroids in mammals is followed by tetanic convulsions. This led to the conclusion that certain tetany-producing toxins are formed in the body, which in normal animals must be either removed or turned into non-toxic substances by the parathyroid glands. The origin of these toxic substances, however, is unknown. Though tetanic convulsions and other symptoms of tetany have been reported repeatedly to occur after injection of thymus extracts, in the frog as well as in mammals,[1] no attention has been paid to this fact as possibly containing the solution of the problem in which organ the tetany-producing substances might be manufactured. The experiments presented in this article seem to indicate that they are contained in and produced by the thymus gland. This harmonizes with the well known fact that tetany is a disease of infants.

In the spring of 1916, about thirty salamander larvæ of the species *Ambystoma maculatum*[2] were fed exclusively on calf's thymus. Each single larva after some time began to suffer from severe tetanic attacks. Since the larvæ of salamanders do not possess parathyroids this observation seemed to be of considerable interest, and, in the fall of 1916, calf's thymus was fed to larvæ of another species of sala-

[1] See Biedl, i, 279, 301 ff.

[2] In the terminology to be employed in the classification of amphibians the nomenclature as worked out by Stejneger and Barbour n their new check list was applied here. According to this the old species *Amblystoma punctatum* corresponds to the new species *Ambystoma maculatum*.

manders, *Ambystoma opacum;* again each single individual after a certain time came down with tetanic attacks. In 1917, the experiments were repeated with the same result on both species. It is, therefore, evident that the thymus gland contains a substance which ·produces tetanic convulsions in the larvæ of *Ambystoma maculatum* and *opacum*.

Tetanic Symptoms.

The tetanic symptoms exhibited by salamander larvæ fed with thymus closely resemble the symptoms produced by parathyroidectomy in mammals.[3] The first attacks in the larvæ are confined to the hind portions of the body. In general, this part of the body suffers more than any other during the entire tetanic period, and when the acute attacks become less and less frequent and severe it is again the hind portions of the body which alone are attacked. Within several days after the onset of acute tetany the entire muscular system exhibits severe clonic convulsions. Each single attack begins at the tail and spreads toward the head; the tremors are severest in the legs and in the muscles of the lower jaw. ˊDuring each attack the mouth is thrown widely open. When the attack begins the animals are thrown on one side. Besides the clonic convulsions a tonic spasm of the entire body is observed; the legs are stretched out and the body is bent with its concavity towards the back. During the attack the animals discharge much air and the vessels of the skin become very red.

In the early stages of the disease the convulsions are induced only upon stimulation which is best effected by removing the larvæ from the water, placing them on filter paper, and pinching the legs or the tail slightly. Each attack lasts only a short time, the larvæ recovering after from 2 to 3 minutes. Later any attempt of the larvæ to swim or to snap at a piece of food suffices to induce an attack and some individuals may float for several days on one side, being rigid from tonic spasm.

8 to 14 days after the first attack the hind legs begin to show signs of a permanent tonic spasm; the legs are stretched backward and become twisted around their longitudinal axis with the inner surface pointing upward; the feet follow this movement and finally the fore legs undergo a similar change.

[3] See Biedl, i, 79 ff.

Relation between Tetany and Development.

Tetany cannot be produced by thymus feeding before a certain developmental stage is reached by the larva; namely, that at which probably the secretive power of the animal's own thymus glands is established. This stage of development corresponds to that of the full development of the toes of the larvæ.

Six larvæ of *Ambystoma opacum* (*opacum*, 1917, B), from North Carolina, hatched in the laboratory from eggs of the same female, were kept at approximately 25°C. and fed on calf's thymus from the 19th day after hatching. The animals were tested for tetany regularly on a definite day every week, for the purpose of recording the percentage of animals suffering from tetany; clonic convulsions of the entire body as well as of the hind portion only were considered as indication of tetany. No animal suffered from tetany when examined the first time after the commencement of the thymus feeding. With the progress of development (as indicated by the development of the toes) the percentage of animals suffering from tetanic convulsions increased and reached a first maximum at the time when all toes were developed. No animal had convulsions until all the toes of the fore legs and at least the third toe of the hind limbs were developed.

In a second series of experiments, with eight *Ambystoma opacum* larvæ (*opacum*, 1916, B) hatched from eggs of the same female and kept at approximately 25°C., thymus feeding was started on the 14th day after hatching. None of the larvæ of this series showed any signs of tetany before the full number of toes had developed. In a third series (*Ambystoma opacum*, 1916, D) consisting of eight larvæ from eggs of the same female and of the same age as the larvæ of the previous series, this becomes still more evident. Though the larvæ of Series D, 1916, were fed on thymus simultaneously with Series B, 1916, convulsions developed 4 weeks later than in Series B, 1916. Development was retarded by keeping the larvæ in low temperature (15°C. approximately).

In *Ambystoma maculatum* similar results were obtained. In one instance (*Ambystoma punctatum*, Series K, 1917) six larvæ, all hatched from eggs of the same female and kept at approximately 25°C., were fed on thymus exclusively from the 13th day after hatching. The first acute attacks were observed 5 weeks after hatching.

Similarly it was found in *Ambystoma opacum* as well as in *Ambystoma maculatum* that the acute tetanic attacks disappear entirely after a certain stage is reached, though the animals continued to be fed exclusively on thymus gland. When the larvæ reach a stage in which they resemble normal larvæ ready for metamorphosis, it becomes more and more difficult to induce an acute tetanic attack and no animal ever showed such attacks after it had metamorphosed, in spite of thymus feeding.

Thymus Gland Contains a Tetany-Producing Substance.

The question arises: Why does the thymus gland when fed to salamander larvæ cause tetany only during a certain developmental period, and why is it ineffective before and after this period? In order to give an answer to this question it was necessary to make a histological examination of the larvæ.

As regards the beginning of tetany several larvæ which had been fed thymus were examined histologically just before and after the beginning of tetany. In a number of *Ambystoma opacum* larvæ of the same age and hatched from eggs of the same female (*Ambystoma opacum*, 1917) tetany was observed in a few individuals as early as 4 weeks after hatching; in these few individuals, however, it did not reappear until 6 to 7 weeks after hatching, and at this time the majority of the larvæ developed tetany. At this time larvæ of this set with and without tetany were killed and preserved for examination to find in which point they differed. A preliminary microscopical study of the anatomy and histology of one tetanic and two pretetanic larvæ demonstrated a noteworthy difference in regard to the developmental state of the thymus glands, but in no other respect.

It was found that the larvæ which had not yet developed tetany had smaller thymus glands than the larva which had already fallen a victim to tetany. The thymus glands of the latter larva are more than twice as large as those of the pretetanic larvæ, though the difference in length of the entire animals is only 33 per cent, the tetanic specimens being the larger ones. This is shown in Fig. 1 in which the three left thymus glands of a pretetanic larva (left vertical row)
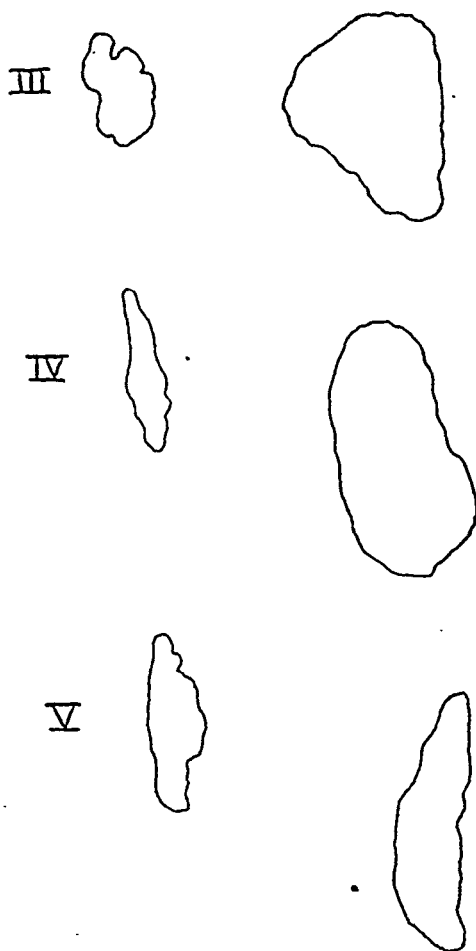
FIG. 1. Comparison between size of the primordial thymus of a pretetanic larva of *Ambystoma opacum* (left vertical row) and a larva of the same species shortly after the beginning of acute tetanic convulsions (right vertical row). It will be seen at a glance that the thymus glands of the latter animal are much larger than those of the non-tetanic larva. The drawings were made with a camera lucida at 490 linear magnification from the largest cross-section through each of the glands.

and of the tetanic larva (right vertical row) are drawn at the same magnification.[4]

Still more significant. are the differences in structure. In the pretetanic larvæ the thymus glands represent small accumulations of purely epithelial cells. They do not show any kind of differentiation. Their epithelial character is indicated also by the presence of considerable quantities of pigment granules such as are found in the epithelium of the pharyngeal pouches from which the glands have separated. In most of the glands there are no mitoses to be found at all, while in some there are one to two mitoses present. No membrana propria is formed.

Compared with the thymus of the pretetanic larvæ, in which none of the characters of a true thymus has developed, five of the glands of the tetanic larva show some of the aspects of a real thymus in as far as they contain elements which are characteristic of that organ. Again no membrana propria is formed, but the glands consist of a variety of cells. Leaving aside the question as to whether or not the newly developed cell elements are of mesenchymal origin,[5] it is sufficient to notice that several kinds of cells now constitute these glands; two of them are recognized clearly as the main elements making up the fully developed thymus of an *Ambystoma opacum* larva.

---

[4] The first and second pairs of the thymus glands have disappeared already in this stage from both the pretetanic and tetanic larvæ, and only the third, fourth, and fifth pairs are present. According to Maximow and to Baldwin the first and second pairs are found absent already in larvæ of from 11.5 to 13.0 mm. in length.

[5] Concerning the development of the amphibian thymus glands and the origin of their cell elements see the papers by Maximow, by Dustin, and by Baldwin. It should be mentioned that the development of the thymus glands in *Ambystoma opacum* was found by the writer to correspond in the main with the description as given by Maximow and Baldwin for *Ambystoma tigrinum* and *Ambystoma maculatum* respectively. The size of the larvæ, however, corresponding to the various developmental stages of the glands was found to be much more variable and in general larger. In a normal stock animal of *Ambystoma opacum*. for instance, the right gland of the third pair was found to be still connected by an epithelial stalk with the brancho-pharyngeal epithelium and of purely epithelial character, though the larva has a length of over 37 mm., while Maximow and Baldwin found the glands fully separated in larvæ of from 10 to 12 mm.; Maximow saw the commencement of mesenchymal immigration in larvæ of about 10 mm. in length, and Baldwin in larvæ of 19 to 20 mm in length.

Correspondingly the epithelial character of the glands is lost; this finds its expression also in the fact that pigment granules have disappeared altogether. In addition a considerable number of mitoses may be found, which mostly seem to be cell divisions of the newly arrived elements. Of particular importance is the fact that in one of the glands of the fifth pair, which I found to be frequently ahead of the third and fourth pairs in development, the cell plasma of a number of cells stains pink, and here one nucleus which also is stained pinkish exhibits a swollen appearance, characters which are ascribed by Maximow to the formation of the medulla of the thymus.

The thymus glands of the pretetanic larvæ are therefore mere accumulations of epithelial cells and do not possess any structures to indicate that they would be capable of exerting any of the functions of a thymus, while in the tetanic larva at least some of the glands resemble a true thymus and probably have commenced to behave as such with regard to function. From these observations we may conclude that the thymus feeding does not begin to call forth tetany in salamander larvæ until the thymus glands of the larvæ themselves are able to secrete. This suggests that tetany is the effect of the combined action of the secretion of certain substances by the thymus glands of the larvæ and of substances introduced into the larva by feeding it with the thymus gland from other animals.

Prevention of Tetany by the Parathyroids.

It is well known that salamander larvæ do not possess parathyroids; these glands develop during metamorphosis. Though the end of the tetanic period coincides approximately with the development of the parathyroid glands, yet larvæ which had ceased to show convulsions for some time apparently possessed no parathyroids when examined histologically. It is, however, possible that in spite of this in such larvæ certain processes had commenced which would soon have led to the formation of these glands and that these processes were already able to check tetany.

A connection between the development of the parathyroids and the cessation of tetany seems probable on account of the fact that tetany

was never observed after metamorphosis and after the development of the parathyroids. This suggestion finds support in the fact that in the larvæ of frogs and toads, which develop their parathyroids soon after hatching, thymus feeding never produces tetanic convulsions.

A Second Mechanism to Counteract the Action of the Thymus in Salamander Larvæ.

If the parathyroids are responsible for preventing tetany in Ambystoma opacum and Ambystoma maculatum, the question may be asked why the larvæ of these salamanders are not normally victims of tetany, since they possess six well developed thymus glands and no parathyroids. It is necessary to assume that the larvæ must possess some other mechanism capable of inhibiting the action of their own thymus glands but inadequate when an excess of thymus substance is introduced into the larva.

That such a mechanism is present becomes evident from the behavior of the larvæ of a third salamander species (Ambystoma tigrinum) closely related to the two others. It is surprising that the larvæ of Ambystoma tigrinum never develop tetany even if they are fed exclusively on thymus gland of which they eat enormous quantities. Ambystoma tigrinum larvæ were fed from about the 14th day after hatching, on calf's thymus exclusively; some of them were kept at 25°C., others at 15°C. One of these larvæ has not metamorphosed yet, 60 weeks after hatching; it has been fed on thymus for more than a year. None of these larvæ ever showed any signs of tetany.

Yet the larvæ of the species Ambystoma tigrinum, like those of the two other species, do not possess parathyroids. Evidently they must have some other mechanism to counteract the tetanic effect of the thymus; and this mechanism must be sufficient not only to prevent tetany from the animal's own thymus but also tetany from an excess amount of thymus when introduced with its food.

CONCLUSIONS.

From the facts stated in this paper it is evident that the thymus gland of mammals contains a substance which is capable of producing

tetany when fed to the larvæ of certain species of salamanders (*Ambystoma opacum* and *Ambystoma maculatum*). As long as the larvæ have not developed their own thymus glands, they are able, by means of some mechanism, to counterbalance the tetanic action of the thymus substance introduced in their food. When, however, the secretion from their own thymus glands is added to the thymus material introduced with the food, this mechanism of preventing tetany becomes inadequate and tetany ensues. In the larva of a third species of salamander, *Ambystoma tigrinum*, this mechanism will prevent tetany even when the larvæ are fed on thymus.

In mammals the parathyroids are known to prevent tetany and are supposed either to absorb the tetany-producing substance and thus prevent its action or to change it into another non-toxic substance. It is at least probable that in the amphibians the parathyroids play the same rôle. Larvæ of anuran amphibians, which develop their parathyroids soon after hatching, never show tetanic convulsions if they are fed on thymus, but in certain species of salamanders, whose parathyroids develop only during metamorphosis, the larvæ invariably have tetanic convulsions upon thymus feeding, while the metamorphosed animals never show tetany.

But in addition to the parathyroids the salamanders must possess still another mechanism which during the larval period inhibits the production of tetany by the animal's own thymus glands. In the larvæ of *Ambystoma opacum* and *Ambystoma maculatum* this mechanism is sufficient only to prevent tetany from the animal's own thymus, while in the larvæ of *Ambystoma tigrinum* it is capable of preventing tetany even when the larvæ are fed with thymus.

If the thymus is the organ by whose action tetany is produced, we can understand why tetany in human beings occurs far more frequently in children than in adults, since in the latter the thymus gland is replaced, at least to a great extent, by connective tissue. The relation of thymus to tetany may also possibly explain the occurrence of tetany during pregnancy; while the parathyroids of the mother may be sufficient to prevent tetany from her largely atrophied thymus, they may not be sufficient to prevent tetany from the excess of thymus substance furnished by the fetus to the blood of the mother.

BIBLIOGRAPHY.

Baldwin, F. M., Pharyngeal derivatives of *Amblystoma*, *J. Morphol.*, 1917–18, xxx, 605.

Biedl, A., Innere Sekretion, Berlin, 2nd edition, 1913.

Dustin, A. P., Recherches d'histologie normale et expérimentale sur le thymus des Amphibiens Anoures, *Arch. biol.*, 1913, xxviii, 1.

Maximow, A., Untersuchungen über Blut und Bindegewebe. IV: Ueber die Histogenese der Thymus bei Amphibien, *Arch. mikr. Anat., 1te Abt.*, 1912, lxxix, 560.

Stejneger, L. H., and Barbour, T., A check list of North American amphibians and reptiles. Cambridge, 1917.

FURTHER PROOF OF THE EXISTENCE OF A SPECIFIC TETANY-PRODUCING SUBSTANCE IN THE THYMUS GLAND.

By EDUARD UHLENHUTH.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

In the preceding note[1] it has been shown that salamander larvæ which are fed exclusively on calf's thymus gland develop tetanic convulsions. Several facts were mentioned supporting the idea that this phenomenon is identical with the tetany observed in mammals after parathyroidectomy; it was also made probable by our experiments that the tetanic convulsions, produced in the salamander larvæ, were due to the action of a specific substance contained in the thymus, inasmuch as the thymus feeding did not become effective until the primordial thymus glands of the larvæ had reached a stage in which they assume some of the structures of true thymus glands.

Instead of assuming that the thymus gland secretes a toxin causing tetany, it might be argued that the effects of thymus feeding were due to the absence from the thymus of some substances which are necessary to make up a complete diet for the salamander larvæ, for in the experiments reported in our previous paper the larvæ were fed exclusively on thymus. In other words, it might be argued that the tetany produced in our experiments was in reality a deficiency phenomenon.

While formerly it was generally assumed that the thymus belongs to the glands with internal secretion, doubts have recently arisen in this regard. After it had apparently been demonstrated by some authors that the thymus contains substances which stimulate the growth of tadpoles of frogs and toads[2] in a specific way, Swingle[3] has

---

[1] Uhlenhuth, E., *J. Gen. Physiol.*, 1918, i, 23.

[2] Gudernatsch, J. F., *Arch. Entwcklngsmechn. Organ.*, 1913, xxxv, 457; *Am. J. Anat.*, 1913–14, xv, 431.

[3] Swingle, W. W., *J. Exp. Zool.*, 1917–18, xxiv, 521.

recently published feeding experiments performed on tadpoles of frogs which seem to prove that the feeding of such larvæ with thymus gland of mammals has no influence whatever on growth. The writer's[4] own experiments on salamanders demonstrate clearly that during the larval period of these animals, thymus when given as food may considerably change the rate of growth, but that this is not due to a specific influence of that gland upon growth.

It is, therefore, of interest that we are able to show that the tetany produced in thymus-fed salamander larvæ is not due to a deficiency in the thymus of one or several substances, but is due to the presence in the thymus of a specific tetany-producing substance.

EXPERIMENTAL.

If tetany in salamander larvæ fed on thymus is due to the absence from the thymus of one or more substances, it is clear that the addition of a sufficient amount of normal food to the thymus diet should prevent the development of tetanic convulsions. It was found, however, that this is not the case.

Thirty larvæ of the species *Ambystoma maculatum*—from the same mother and of the same age—were fed on thymus from about the 5th week after hatching. The larvæ developed tetany very soon; convulsions were noticed as early as 15 days after the first thymus meal. At the end of the 3rd week, the series was divided into two lots, each containing fifteen individuals. One group was fed on thymus exclusively, the other one was fed alternately one day on thymus and one day on earthworms, the latter constituting a complete diet in the case of the salamanders.

The result of this experiment was that tetany is not stopped by the addition of a sufficient amount of complete diet, but on the contrary developed still further towards a maximum. Therefore, the tetanic convulsions resulting from thymus feeding are not due to a deficiency in the diet but must be due to a specific tetany-producing substance contained in the thymus gland.

It was also noticed that in the mixed food series the tetany period was shorter than in the thymus series and that the percentage of ani-

[4] Uhlenhuth, *Proc. Soc. Exp. Biol. and Med.*, 1917, xv, 37.

mals suffering from tetany was smaller on a mixed diet than on pure thymus diet. This is due to several factors.

First, it was pointed out in a previous article[1] that the salamander larvæ, though they have no parathyroids, must possess some other mechanism capable of doing away with a certain limited amount of the tetany-producing poison; otherwise even normal larvæ not fed on thymus should show tetany as soon as their own thymus glands are sufficiently developed. In one species (*Ambystoma tigrinum*) this mechanism is strong enough to prevent tetany even if by means of feeding thymus exclusively the body is flooded with a large amount of the tetany toxin. Hence, it is not surprising that if a smaller dose of the toxin is introduced into the body, the larvæ should be capable of removing—or antagonizing—so much of the toxin that the remaining part may be less effective than a maximum dose would be. Hence the fact that tetanic attacks occurred less frequently in each single individual on a mixed diet than on an exclusive thymus diet is due to the difference in the amount of thymus substance.

A second factor is still more important as regards the decrease of tetany in the mixed food series than the first factor mentioned. It has been pointed out in another communication[1] that larvæ of the species of *Ambystoma maculatum* and *Ambystoma opacum* cease to show tetanic convulsions after metamorphosis, probably because of the action of the parathyroids developing during metamorphosis. Metamorphosis started in the mixed food series 7 weeks earlier than in the pure thymus series, and was finished in the former series 19 weeks earlier than in the latter series.

We have alluded to the fact that some authors believe they have demonstrated that thymus feeding retarded the metamorphosis of the tadpoles of frogs and that this effect was specific. This conclusion is not correct. It became clear in the course of our study of amphibian metamorphosis[5] that the retardation of metamorphosis upon exclusive thymus feeding is probably not due to a specific effect of the thymus, but to a deficiency in the diet. If retardation of metamorphosis upon thymus feeding were due only to the absence of certain substances from the thymus and tetany were due to the presence of certain toxic

[5] Uhlenhuth, E., *J. Exp. Zool.*, 1918, xxv, 135.

substances in the thymus, we should expect that upon addition of a complete diet to the thymus food tetany would still occur, but metamorphosis would take place in the normal way and at the normal time. In fact, metamorphosis started even a few days earlier than in a control series consisting of thirty larvæ of the same age.   Thus by adding normal food to the thymus diet the specific actions of the thymus may be sifted out from the non-specific ones.

The tetany produced by exclusive thymus feeding is a specific effect of the thymus due to the existence of a tetany-producing poison in that gland, while the retardation of metamorphosis in the case of an exclusive diet of thymus is due to a deficiency in the diet.

CONCLUSIONS AND SUMMARY.

The effect of the thymus gland in producing tetany is due to a specific tetany toxin produced by and contained in the thymus, and the thymus gland must be added to the group of glands for which the function of internal secretion has been demonstrated.

DIFFERENCE IN THE ACTION OF RADIUM ON GREEN PLANTS IN THE PRESENCE AND ABSENCE OF LIGHT.

By CHARLES PACKARD.

(From the Marine Biological Laboratory, Woods Hole.)

Experiments by Willcock[1] on the effect of radium radiations on *Hydra viridis* and *Hydra fusca* show that the green form is far more resistant to rays than the brown. The green hydras are not killed by an exposure of $4\frac{1}{2}$ hours to 50 mg. of radium bromide; the brown hydras, on the other hand, die after "comparatively short exposures." I have repeated this experiment several times and have obtained the same results. Willcock also found that protozoa containing chlorophyll are uniformly more resistant than those which have none. Since these experiments were carried on in daylight, it seemed possible that the difference in the reaction between the two classes of animals experimented upon might be due to the activity of the chlorophyll.

If this assumption is correct, we should expect that plant cells radiated in complete darkness, when the chlorophyll is not active, would be more sensitive to the rays than in the light. To test this point I have made a number of experiments on *Spirogyra* and *Volvox*.

The radium (20.4 mg. of element, enclosed in a thin-walled silver tube) was placed in a watch-glass filled with water, and the *Spirogyra* filaments were laid across it. In the light, the cells nearest the tube began to show a typical phenomenon of disintegration in 2 to 3 hours. This disintegration consists in the following.

The chlorophyll bands first contract somewhat, losing their spiral arrangement. A few minutes later they resolve into an irregular heap of chlorophyll in one end of the cell. In the light, this phenomenon began to be noticed after an exposure of from $2\frac{1}{2}$ to 3 hours. The

[1] Willcock, E. G., *J. Physiol.*, 1904, xxx, 449.

disintegration became visible in many cells simultaneously, and within 5 minutes after the disintegration began to be visible in a few cells, practically all the cells in the vicinity of the radium were thus affected.

In the dark, this phenomenon of disintegration commenced in about one-fourth of the time; namely, after an exposure of from 35 to 70 minutes, the average being 45 minutes.

Colonies of *Volvox* were exposed in a small glass tube in which the radium preparation was placed. In the light, the colonies became motionless in 2 hours; in the dark, the same condition was attained in 55 minutes. These periods represent the average of many trials.

It is very evident that the life of the cell is prolonged by some condition connected with photosynthesis; a more definite statement cannot be made on the basis of these preliminary experiments.

AMPHOTERIC COLLOIDS.

I. CHEMICAL INFLUENCE OF THE HYDROGEN ION CONCENTRATION.

By JACQUES LOEB.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

I.

It is often stated (Pauli, Höber, and others) that both ions of a neutral salt affect the physical properties of an amphoteric electrolyte (ampholyte) simultaneously and in the opposite sense; so that the total result is the algebraic sum of the opposite action of the oppositely charged ions of the neutral salt.[1] The writer has been able to show by experiments on gelatin and on pig's bladder that this statement is not correct, but that apparently only one of the two ions of the neutral salt acts upon the ampholyte; namely, the one which has the opposite electric charge from that of the ampholyte.[2]

The hydrogen ion concentration of the gelatin solution used by the writer was 10^{-7} (or in Sörensen's logarithmic symbol pH = 7.0) and this gelatin reacted with neutral salts as if it were an anion capable of combining with the cation of a neutral salt. When, however, powdered gelatin is treated for some time with an acid, *e.g.* HCl, gelatin chloride (or hydrochloride) is formed, which is supposed to dissociate electrolytically into a positive gelatin ion and a negative chlorion. The writer found that when the supernatant

---

[1] This and other erroneous statements current in colloid chemistry are due to the fact that the previous investigators always studied the action of neutral salts upon proteins in the presence of the salt. The writer removed these salts after they had a chance to act on the gelatin. This procedure is necessary on account of the fact that the manifestation of the effect of the electrolyte upon the gelatin is repressed in the presence of the electrolyte.

[2] Loeb, J., *J. Biol. Chem.*, 1917, xxxi, 343; 1918, xxxiii, 531; xxxiv, 77, 395, 489; 1918, xxxv, 497.

acid is washed away from such powdered gelatin it is affected only by the anion of a neutral salt, while the cation of the salt used has apparently no effect.

The writer found that when powdered gelatin is treated for some time with a base, *e.g.* NaOH, the gelatin (after the supernatant NaOH is washed away) is only capable of combining with the cations of neutral salts, while the anions of neutral salts are without effect.

It seemed necessary to find out where the turning point for these two different modes of reaction lies, whether at the point of neutrality of the solution or at the isoelectric point of gelatin. The fact that gelatin solutions at the point of neutrality (pH = 7.0) react with neutral salts as if they had previously received an alkaline treatment, suggests that the turning point lies at a more acid reaction of the gelatin and since the isoelectric point lies at a more acid reaction it was *a priori* likely that the isoelectric point for gelatin might be the turning point.

Amphoteric electrolytes are defined by the fact that they can dissociate electrolytically as acids as well as bases. When they dissociate as acids they are supposed to send more H ions than OH ions into solution and the ampholyte ion migrates in the electric field to the anode. When they dissociate as bases they are supposed to send more OH ions than H ions into solution, and the ampholyte migrates in the electric field as a cation. At the isoelectric point the ampholyte is supposed to send out an equal number of H and OH ions and it migrates in neither direction. Gelatin is naturally a stronger acid than base and hence migrates to the anode. By increasing the hydrogen ion concentration of the surrounding solution through the addition of some acid the dissociation of gelatin as acid can be repressed to the level of its dissociation as a base. This is the isoelectric point at which it migrates in neither direction and at which the total number of ions it sends into a solution is a minimum. With the addition of more acid it migrates to the cathode. Michaelis determined the isoelectric point of gelatin solutions by the migration method and found it in the neighborhood of $C_H = 2.10^{-5}$ (or in Sörensen's logarithmic symbol pH = 4.7).[3] Since the solution of gelatin used had a pH of 7.0 it was on the alkali side of the isoelectric point. We shall

[3] Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914.

show in this paper that gelatin on the alkali or less acid side of its isoelectric point can combine only with the cations of neutral salts, as we had actually found empirically in our previously published papers.

Moreover, it will follow from the experiments to be published in this paper that the gelatin ion can dissociate electrolytically only as one of the two, either as a cation or as an anion but not as both simultaneously (except to a negligible degree), and that the hydrogen ion concentration of the solution alone determines in which of the two ways it dissociates. We shall also show that the isoelectric point is not only the point where the gelatin sends out as many H as OH ions but that it is the point where it apparently sends out neither; *i.e.*, where it cannot exist in an ionized condition at all.

II.

Our method of testing whether gelatin reacts with the cation or the anion of a neutral salt is based on the difference of the effects of univalent and bivalent ions upon gelatin. It was found in the writer's experiments on osmotic pressure, viscosity, alcohol precipitation, and swelling that a salt of the type Na_2SO_4 (univalent cation, bivalent anion) of a certain molecular concentration, *e.g.* $M/128$, has quantitatively and qualitatively the same effect upon gelatin (pH = 7.0) as a salt of the type NaCl at the molecular concentration $M/64$; *i.e.*, twice the molecular concentration of the Na_2SO_4.[4] This proves that only the cation of the salt and not the anion acts upon the gelatin. It was found, moreover, that all neutral salts with monovalent cation (Li, Na, K, NH_4) cause an increase in osmotic pressure, viscosity, alcohol number, and swelling of gelatin, while salts with a bivalent cation (Mg, Ca, Sr, Ba, Co, Mn) cause no such increase; and if a salt with bivalent cation is added in small quantity to a large quantity of a salt with univalent cation, the increase in osmotic pressure, viscosity, alcohol number, and swelling caused by the salt with univalent cation is inhibited (antagonistic salt action).[5] The same is

---

[4] Loeb, *J. Biol. Chem.*, 1918, xxxiv, 77, 489.

true for gelatin previously treated with NaOH. When such gelatin is subsequently treated with a salt with univalent cation its viscosity, osmotic pressure, swelling, etc., are increased, while when it is treated with a salt with bivalent cation this increase does not occur and its previous osmotic pressure, etc., are diminished. In all these cases the gelatin migrates as an anion, and the compounds formed are metal gelatinates where the metal is that of the alkali or salt used.

On the other hand, if gelatin has first been treated with an acid, *e.g.* HCl, gelatin chloride is formed which shows an increase in osmotic pressure, swelling, viscosity, and alcohol number. That such a gelatin (which is freed from the original acid by washing) reacts only with the anion of neutral salts is proved by the fact that a solution of a salt of the type $Ca(NO_3)_2$ (univalent anion, bivalent cation) influences gelatin chloride quantitatively and qualitatively like a salt of the type $NaNO_3$ (univalent anion, univalent cation) of half the molecular concentration; *e.g.*, $M/128$ $Ca(NO_3)_2$ acts like $M/64$ $NaNO_3$.[4] This is only possible if the anions of the neutral salt react with the gelatin chloride, while the cations have no effect, since otherwise the depressing effect of Ca mentioned before should be noticeable, which is not the case. If gelatin chloride freed from the excess of acid by washing is treated with a sufficiently high concentration of a neutral salt with univalent anion the osmotic pressure, swelling, viscosity, etc., of the gelatin increase after the salt is washed away. If, however, the anion of the salt used is bivalent, *e.g.* sulfate, oxalate, succinate, it causes less increase or an actual decrease of the osmotic pressure, viscosity, alcohol number, and swelling of the gelatin.

Hence by making use of the depressing effects of bivalent ions and the opposite effect of the univalent ions we can determine at which pH the gelatin begins to combine with the cation and ceases to combine with the anion of a neutral salt. Our experiments show that this critical point lies for gelatin at a hydrogen ion concentration 2.10^{-5} (pH = 4.7), *i.e.* the isoelectric point of gelatin, and not at the point of neutrality.

[5] All these effects were measured after the excess of the electrolyte was washed away (see footnote 1).

Our method was the same as in our previous experiments. 1 gm. of finely powdered gelatin was put into each of a series of beakers containing 100 cc. of a solution of HCl of various concentrations (M/8 to M/8192). This series served as a control. We then experimented with HCl solutions of different concentrations (M/8 to M/8192), each containing a certain salt (e.g. Na₂SO₄ or Ca(NO₃)₂) in the same concentration (M/16). The gelatin remained for 30 minutes in these solutions of a temperature of 20°C. The gelatin was then poured into cylindrical funnels of the same diameter, to allow the acid or acid-salt mixture to drain off, and the gelatin was then perfused three times with 25 cc. of distilled water of 10°C., and a fourth time with H₂O of 20°C.[6] The gelatin was then melted and made into a 1 per cent solution. Dialysis experiments, titration experiments, and determinations of pH by Sörensen and Clark's indicator method[7] made it probable that the solutions contained no acid except that due to hydrolytic dissociation of the gelatin salt formed; it was also possible to show that the neutral salt added to the HCl solution had been washed away.

The following physical qualities of the washed gelatin were ascertained:

1. The conductivity $\left(\dfrac{10,000}{\text{ohms}}\right)$. at 24°C.

2. The osmotic pressure expressed in mm. height of the 1 per cent gelatin solution in the manometer tube.

3. The total swelling expressed in mm. of the height of the cylindrical mass of gelatin.

4. The alcohol number; i.e., the cc. of 95 per cent alcohol required to precipitate 5 cc. of 1 per cent gelatin solution at 20°C.[5]

The results are given in the form of curves in which the logarithms of the concentration of the acid used are the abscissæ and the above mentioned values for conductivity, osmotic pressure, swelling, and alcohol number, the ordinates. The pH of each gelatin solution was

[6] The water was used at these temperatures to prevent excessive swelling and a retardation of the process of filtration.

[7] We are obliged to Dr. Dernby for demonstrating to us the use of these methods.

[5] The viscosity of the solutions was determined in a special set of experiments and the viscosity curves ran parallel to those for osmotic pressure. They may therefore be omitted in this paper.
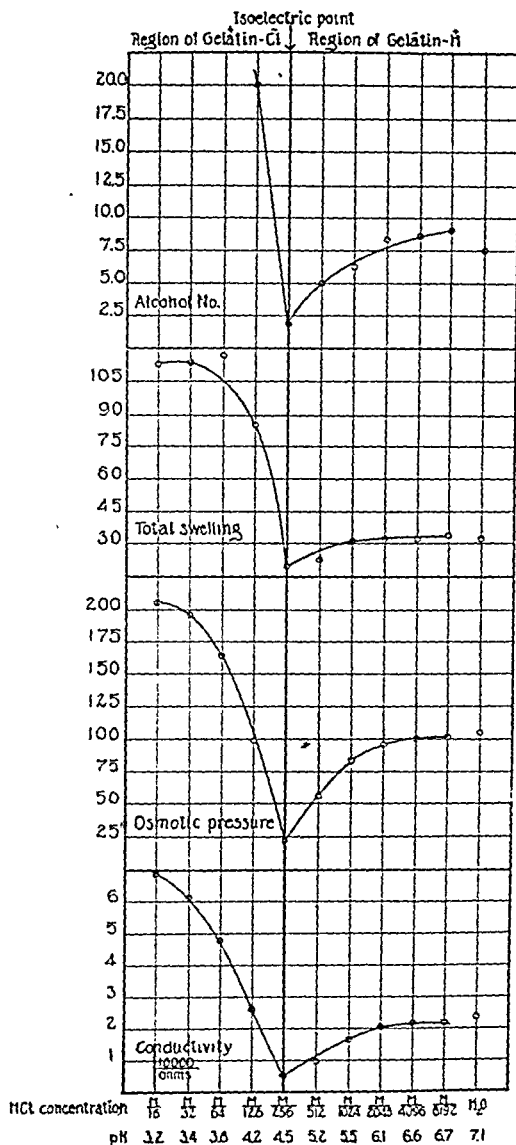
FIG. 1. HCl curves. Curves of the conductivity, osmotic pressure, swelling, and alcohol number of gelatin previously treated with various concentrations of HCl and then freed from excess of HCl by washing with water. On the axis of abscissæ are the logarithms of the HCl concentration used, and under each concentration is the pH of the 1 per cent gelatin solution in H_2O (made from the washed gelatin). The ordinates are the values for conductivity, osmotic pressure, swelling, and alcohol number for the gelatin. In the region of the isoelectric point (pH = 4.7) all the curves have a minimum. On the left of this point gelatin exists in the form of gelatin chloride (with high ionization), the curves rising more rapidly than on the right of the isoelectric point where the gelatin exists in the form of common gelatin which dissociates as a very weak acid. All four curves are nearly parallel.

ascertained and is put under the figures for the logarithms of the concentration.

We now will in turn discuss the four main groups of curves.

Fig. 1 gives the curves for gelatin treated for 30 minutes with HCl alone and then freed from the supernatant and intercapillary acid (with the exception of that formed by hydrolysis of gelatin chloride). The striking fact is that the conductivity, osmotic pressure, swelling, and alcohol number (and viscosity, though this curve is omitted) have all a minimum near a point where the hydrogen ion concentration pH = 4.7; *i.e.*, at about the isoelectric point as determined for gelatin solution by Michaelis on the basis of migration experiments. We may add that the transparency of the solution was also a minimum at the isoelectric point, where the solution was generally quite opaque after standing over night.

Though it was already known[3] that the viscosity and the swelling of gelatin are a minimum at pH = 4.7, our experiments for the first time furnish the proof that this is also the exact location of the minimum for conductivity, osmotic pressure, alcohol number, and transparency.

The second fact of significance is the sudden rise of the system of curves on either side of the isoelectric point. Since the conductivity curve is the direct expression of the degree of electrolytic dissociation existing in the gelatin solution (which had been freed from all ions except those formed by electrolytic and hydrolytic dissociation of the gelatin salt), these curves may be considered as a function of the degree of electrolytic dissociation of the gelatin.

Gelatin may exist in the form of non-ionized gelatin on both sides of the isoelectric point, but we shall endeavor to show that ionized gelatin exists in a different ionic state on the two sides of the isoelectric point. On the right, less acid side of the isoelectric point it can only exist as a negative ion (*e.g.* gelatin-$\overset{-}{H}$ or gelatin-$\overset{+}{Na}$, etc.), while on the left, more acid side of the isoelectric point it can only exist as a cation (*e.g.* gelatin-$\overset{+}{C}$$\overset{-}{l}$ or gelatin-$\overset{+}{O}$$\overset{-}{H}$). When we study the curves for gelatin treated previously with HCl and freed from the excess of HCl by washing as described, the two branches of the curves on the two sides of the isoelectric point are asymmetrical (Fig. 1).

The left branches rise higher than the right ones on account of the fact that on the left side of the isoelectric point we have gelatin chloride which as a salt has a high dissociation constant and which dissociates into gelatin $\overset{+}{\text{gelatin}}$ and $\overset{-}{\text{Cl}}$, while on the right of the isoelectric point the curves are the expression of the electrolytic dissociation of common gelatin which dissociates like a very weak acid into $\overset{+}{\text{gelatin}}$ and $\overset{-}{\text{H}}$. Hence the limited rise of conductivity on the right and the considerable rise on the left of the isoelectric point.

The parallelism between the curve for conductivity and that for the other physical properties of the gelatin solution is so complete that the idea is unavoidable that the degree of conductivity, *i.e.* of the ionization of the gelatin, determines all the other physical properties of the gelatin solution; the pH only entering to the extent as it influences the possibility and character of the ionization of the gelatin. We shall see presently, however, that this parallelism between the conductivity and the curve for the other physical properties of the gelatin solution does not hold throughout but that in addition to the degree of electrolytic (and hydrolytic) dissociation still another variable enters. The main fact for us is the typical asymmetry of the two branches of the curve to the left and to the right of the isoelectric point, the branch on the left (the more acid side) being steeper and much higher than the branch on the right.

A comparison of the curves of Figs. 2, 3, and 4 with those of Fig. 1 will prove that gelatin when it ionizes will ionize on the right, less acid side of the isoelectric point as an anion, on the left, more acid side of the isoelectric point as a cation.

Fig. 2 represents the effect of the same concentrations of HCl as Fig. 1, except that each acid solution contained so much Na_2SO_4 as to make them all $M/16$ for this salt. We will call this system of curves the Na_2SO_4-HCl curves to distinguish them from the pure HCl curves represented by Fig. 1. The powdered gelatin was put for 30 minutes at 20° into these acid-salt mixtures and then freed from all the supernatant free acid and salt by the process of washing described in the writer's previous papers. If it is true that the isoelectric point is also a chemical turning point and that gelatin can ionize only as an

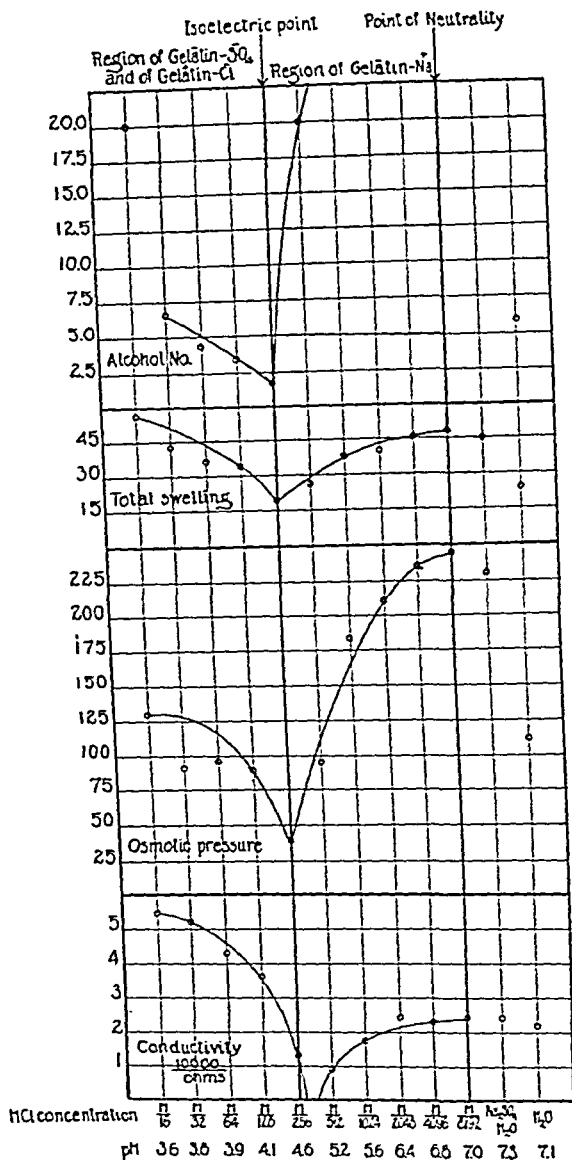Fig. 2. Na_2SO_4-HCl curves. Isoelectric point and minimum again near pH = 4.7. Values for osmotic pressure, swelling, and alcohol number on right of isoelectric point, where gelatin exists as sodium gelatinate, higher than on left, where it exists as mixture of gelatin sulfate and gelatin chloride. The curves for osmotic pressure, swelling, and alcohol number are *not* parallel to the curve for conductivity.

47

anion on the right side of this point, and as a cation on the left side, we should be able to predict the difference between the two systems of curves, namely the HCl curves and the Na_2SO_4-HCl curves (Figs. 1 and 2), as far as osmotic pressure, swelling, and alcohol number are concerned. On the right, less acid side of the isoelectric point gelatin can dissociate only as a negative ion and hence, reacting with Na_2SO_4, it should be transformed into sodium gelatinate. On the left side, where gelatin can only exist as cation, in a mixture of HCl and Na_2SO_4 gelatin chloride and gelatin sulfate should be formed. In the Na_2SO_4-HCl curves (Fig. 2) we should, therefore, expect to find on the right side of the isoelectric point (pH > 4.7) sodium gelat- inate, showing a high osmotic pressure, a high alcohol number, etc. On the left side of the isoelectric point where gelatin exists as gelatin chloride and gelatin sulfate, we should find lower values for osmotic pressure, alcohol number, etc., since we have shown in former experiments that the addition of SO_4 to Cl represses the increase in osmotic pressure, viscosity, swelling, caused by Cl. Hence we should expect an asymmetry in this system of curves of exactly the reverse kind of that found in the pure HCl experiment. A glance at Fig. 2 shows that this is the case, for osmotic pressure, alcohol number, but not for conductivity and pH, which are the same for the same HCl concentrations as in the pure HCl experiment (Fig. 1). We shall return later to this difference which is of great theoretical importance.

Hence we reach the conclusion that the isoelectric point of the gelatin is also the turning point for the chemical reaction between gelatin and neutral salt, inasmuch as the gelatin on the less acid, right side of the isoelectric point can exist in an ionized state only as a negative gelatin ion capable of combining with cations but not with anions, while on the left (more acid) side of the isoelectric point it can exist in an ionized state only as a positive gelatin ion capable of combining with the anions of a neutral salt.

This conclusion is confirmed by the experiments of the $Ca(NO_3)_2$- HCl series. In this series, represented by the curves of Fig. 3, the same concentrations of HCl from M/8 to M/4096 were used and each HCl solution contained so much $Ca(NO_3)_2$ as to make it M/16 in regard to $Ca(NO_3)_2$. If our theory is correct, the asymmetry of the

curves for osmotic pressure, swelling, and alcohol number should be the reverse for the $Ca(NO_3)_2$ series as that for Na_2SO_4. On the right side of the isoelectric point (for $pH > 4.7$) gelatin should exist in the form of Ca gelatinate which according to our previous observations has a low osmotic pressure, a low alcohol number, low viscosity, etc. On the left side of the isoelectric point it should exist in the form of gelatin chloride and nitrate in which form gelatin has a high osmotic pressure, swells considerably, has a high viscosity, and a high alcohol number. The forms of the curves actually found are represented in Fig. 3, and show that the facts agree with our theory.

Our theory finally demands that the two branches of the curve should become almost symmetrical in regard to the isoelectric point, if we add to the HCl a salt with univalent cation and univalent anion, e.g. NaBr. NaBr was added to the HCl solutions so as to make the solution M/16 in regard to this salt, and Fig. 4 gives the curves for gelatin treated with NaBr-HCl. It is obvious that the branches of the curves to both sides of the isoelectric point are much more symmetrical than was the case in the three other groups of curves. In this case we have on the right side of the isoelectric point Na-gelatinate, on the left a mixture of gelatin bromide and chloride. On both sides of the isoelectric point we have gelatin in combination with univalent ions, which as we have shown in our previous papers yield a high osmotic pressure, a high swelling, etc.

The experiments were repeated with other salts, $Mg(NO_3)_2$, Na_2 oxalate, etc., and our conclusions were confirmed. In the experiments described in this paper the reaction between the anions of the neutral salt with gelatin took place while the gelatin was in the acid solution; while the reaction between the cations of the neutral salt and gelatin could not take place while the gelatin was in the acid solution since all these concentrations were on the acid side of the isoelectric point of gelatin. This reaction must have taken place after the acid treatment and during the process of washing with H_2O when the gelatin previously treated with M/256 HCl had reached a $pH > 4.7$.

This conclusion is confirmed by the experiments in which the gelatin was first treated with acid, then freed from the excess of acid by perfusions with distilled water, and was then treated with a salt and
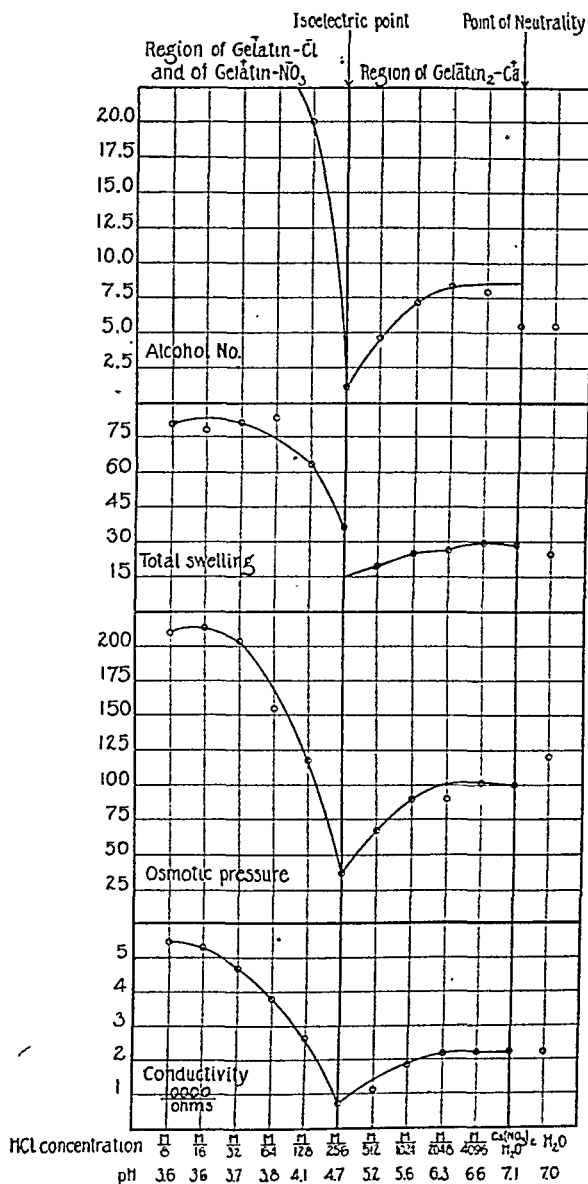
FIG. 3. Ca(NO₃)₂-HCl curves. Minima of all curves again at pH = 4.7 (isoelectric point). The branches of the curves for osmotic pressure, swelling, and alcohol number on the left, where gelatin exists as mixture of gelatin Cl and gelatin nitrate, higher than the branches on right of the isoelectric point, where gelatin exists as calcium gelatinate.

50
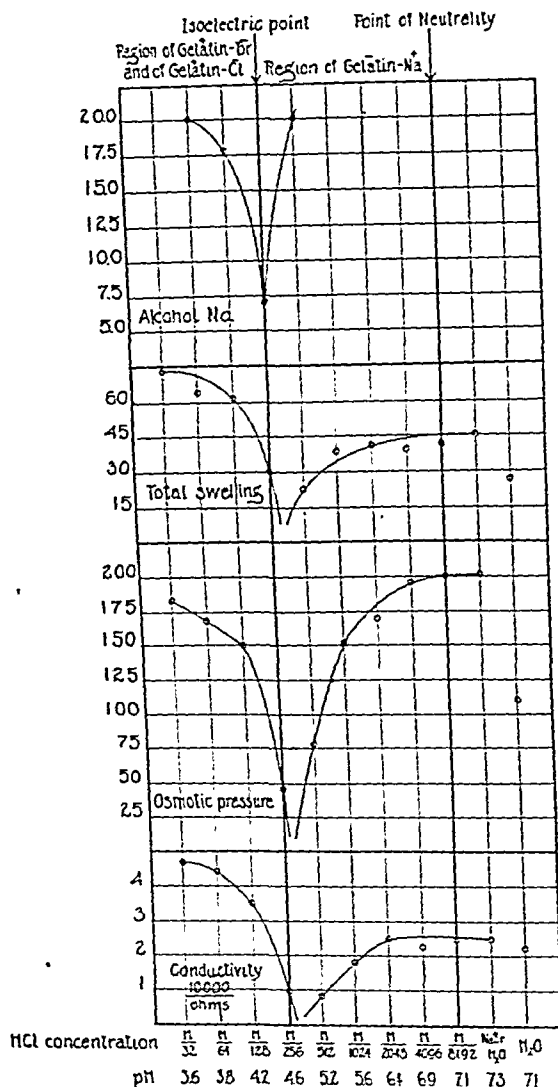
FIG. 4. NaBr-HCl curves. Minima again at pH = 4.7 (isoelectric point). Curves for osmotic pressure, swelling, and alcohol number almost symmetrical in regard to isoelectric point. On left side gelatin chloride and bromide, on right side sodium gelatinate.

then freed again from the salt by perfusion with water.[9] In this case the same results were obtained as those stated in this paper; namely: *When pH of the gelatin solutions is >4.7, the gelatin can exist in an ionized condition only as an anion capable of combining with cations only to form metal gelatinates, while on the more acid side of the iso-electric point (pH <4.7) gelatin can exist in an ionized form only as a cation capable of combining only with the anion of the neutral salt.* This law holds probably for all amphoteric electrolytes. The question then arises: How does an amphoteric electrolyte react with neutral salts at its isoelectric point? Before we answer this question we will. point out an important fact shown by our curves, though this has no direct bearing on the subject under discussion.

III.

A comparison of the curves for the conductivities with those for the other properties of gelatin in Figs. 1 to 4 shows that the curves for conductivity are practically identical in all four figures as are also the values for pH; while the curves for osmotic pressure, etc., differ according to the valency of the ion combining with the gelatin. Since the conductivity curves are the direct expression of the degree of electrolytic dissociation, we can now say that the difference in the effect of univalent and bivalent ions demonstrated in the writer's previous publications is not merely a difference of the degree of ionization of the gelatin but is due to another effect upon the gelatin molecule. The important work by Langmuir and by Harkins on surface tension has suggested if not proved the idea of polarized molecules and of different degrees of solubility for different parts of the same organic molecule. It seems possible from their work that the solubility is confined to certain groups of the gelatin molecule or the protein molecule in general. It is quite possible that this will ultimately explain why the entrance of a univalent anion or cation into a gelatin molecule increases its solubility in water (or its attracting power for water molecules) and with this its osmotic pressure, alcohol ↖ number, viscosity, etc., while the entrance of a bivalent ion (com-

[9] In order not to increase the bulk of this paper we may be pardoned for omitting the details of these experiments.

bining possibly with two or more molecules of gelatin) will not cause or may even prevent such an increase, without altering the conductivity of the gelatin solution. The physical qualities of a gelatin solution depend, therefore, aside from the degree of its electrolytic dissociation and its hydrogen ion concentration upon a constitutional factor varying with the valency of the ion with which the gelatin is in combination. We intend to return to this question in a later publication.

IV.

The writer was curious to find out how gelatin reacts with neutral salts when brought to the isoelectric point. This was done by treating gelatin for 30 minutes with M/256 acetic acid or with M/256 or M/512 HCl, etc., and then perfusing the gelatin repeatedly with 25 cc. of H_2O in the way described. That the gelatin had reached the isoelectric point was ascertained by measurements of pH, of conductivity, osmotic pressure, etc. When gelatin on either side of the isoelectric point is treated with M/4 or M/8 NaCl or any other salt with univalent anion or cation, it shows an increase in osmotic pressure, swelling, alcohol number, after the salt is washed away; when, however, gelatin rendered isoelectric in the way described is treated with the same solution of NaCl, all its physical properties, conductivity, osmotic pressure, swelling, alcohol number, transparency, remain unaltered after the salt is washed away. It is immaterial whether we treat such gelatin with NaCl, Na_2SO_4, $CaCl_2$, or any other neutral salt; and it is also immaterial whether we use high or low concentrations of these salts; the physical qualities of the gelatin remain unaltered. Numerous experiments were made in which the nature of the acid, the concentrations, the number of washings, etc., used to reach the isoelectric point varied, but all yielded the same result, which means that *at the isoelectric point gelatin is chemically inert*, incapable of reacting with neutral salts. When, however, the pH of the gelatin deviates only slightly from that of the isoelectric point by becoming either $>$ or <4.7, the neutral salts react with gelatin in the way described in this and previous papers.

This is exactly what our theory demands, according to which gelatin can dissociate electrolytically only as an anion when pH is > 4.7, as

a cation when pH is < 4.7.   Hence it can dissociate as neither when pH $= 4.7$.   Not being able to dissociate electrolytically, it cannot react chemically with the ions of a neutral salt, as was found to be the case.

This inability to dissociate electrolytically and hence to react with neutral salts at the isoelectric point should be a common property of all amphoteric electrolytes and this seems to be the case.   While this note goes to press a paper has appeared by Van Slyke and Baker,[10] containing the following sentence.   "At the isoelectric point casein combines with neither acid nor base;" *i.e.*, with neither cation nor anion.

V.

The facts shown thus far support the idea that amphoteric electrolytes dissociate in a different way on the two sides of the isoelectric point; on the less acid side of that point they can ionize only as anions capable of combining with the cations of neutral salts, but not with their anions; on the acid side of the isoelectric point they can ionize only as cations capable of combining with the anion of a neutral salt, but not with its cation.   The question then arises: What will happen when we bring a salt like sodium gelatinate in which gelatin is an anion on the acid side of the isoelectric point?   Our theory demands that sodium gelatinate produced by treating gelatin with NaOH should not continue to exist when pH < 4.7, and that in this case gelatin should behave as if it had not been treated with NaOH.   This is what actually happens.   The writer made this experiment some time before he had arrived at the theory published in this paper.   He had undertaken the experiment in the expectation that gelatin first treated with alkali and then with acid should show a superposition of the effects of both treatments.   1 gm. of finely powdered gelatin was put for 30 minutes at 20°C. into each of a series of beakers all containing $M/32$ NaOH.   After this, each powder was put on a filter, the alkali was allowed to drain off, and each filter was perfused four times with 25 cc. of H_2O to remove the last traces of NaOH held in the capillary spaces (except the NaOH formed by the hydrolytic dissociation of the sodium gelatinate).   Then each filter was perfused three

[10] Van Slyke, L. L., and Baker, J. C., *J. Biol. Chem.*, 1918, xxxv, 147.

times with 25 cc. of a HCl solution which varied for the different filters from м/8 to м/8192, and after this each filter was perfused four times with H_2O to remove the HCl not bound by the gelatin.

The result is given in the curves of Fig. 5 for the conductivity, osmotic pressure, swelling, and alcohol number. The curves show a marked minimum at about pH = 4.8; i.e., the isoelectric point. The appearance of this low minimum of the values for conductivity, osmotic pressure, swelling, etc., characteristic of the isoelectric point, was at first a surprise and a puzzle to the writer. On the right side the curve of osmotic pressure rises to a value of 125 to 150 mm. which corresponds to the value for gelatin treated with м/32 NaOH and washed ten times with H_2O (without any subsequent treatment with HCl). On the acid side of the isoelectric point the curve of osmotic pressure rises to that point which corresponds to the effect the same concentration of HCl has on gelatin not previously treated with alkali. Hence the result of the acid treatment is on the left, more acid side from the isoelectric point, exactly the same as when we use gelatin not previously treated with м/32 NaOH and apply to it the same treatment with HCl as in this experiment; while on the right, less acid side of the isoelectric point, it is the same as if the gelatin had been treated with NaOH of increasing concentrations.

This permits of only one explanation, which in the light of the experiments published in the preceding parts of this paper is as follows. Sodium gelatinate, or perhaps more correctly a negative gelatin ion, cannot continue to exist except at hydrogen ion concentrations lower than that defining the isoelectric point. This means that in the acid solutions all or most of the sodium gelatinate must have given off its sodium ions and must have been retransformed into the weak gelatin acid; or some other constitutional (tautomeric?) change must have occurred in the gelatin molecule on the acid side of the isoelectric point. On the alkali side of the isoelectric point the sodium gelatinate could and did continue to exist.

This experiment was repeated with different concentrations of acids and alkalies, all confirming our conclusions. The experiment was also reversed, i.c. the gelatin was first treated with acid and then with alkali, and again our theory was confirmed.

It is of interest to see our view confirmed also by another set of facts. It has long been known that proteins and probably ampho-

Isoelectric point

Region of Gelatin-Cl   Region of Gelatin-Na

20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5

Alcohol No.

75
60
45
30

Total swelling

175
150
125
100
75
50
25

Osmotic pressure

4
3
2
1

Conductivity
$\frac{10000}{\text{ohms}}$

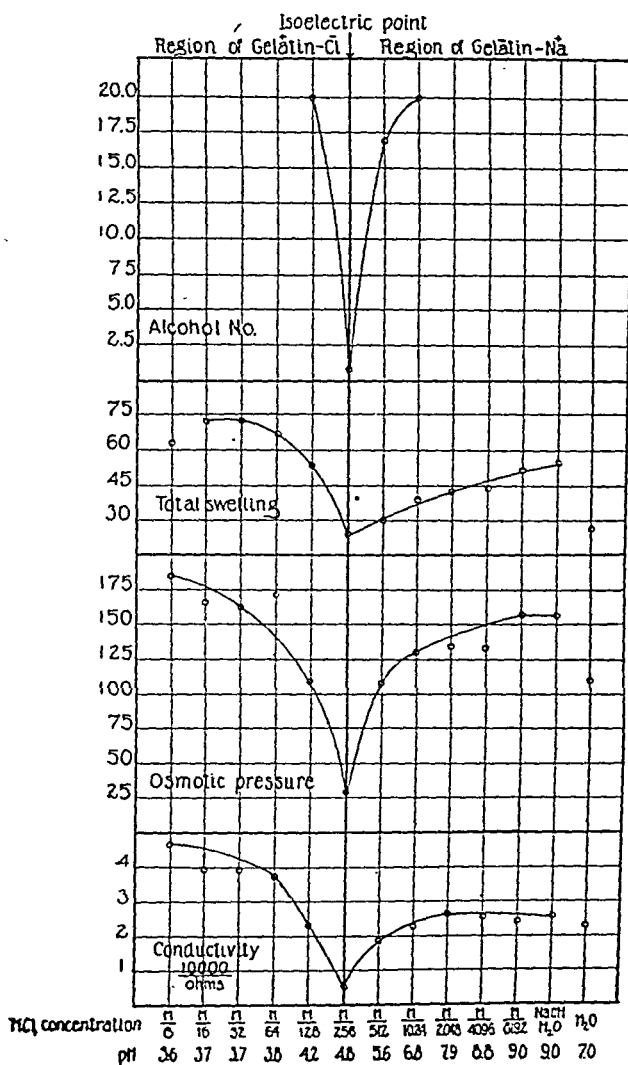| HCl concentration	$\frac{n}{0}$	$\frac{n}{16}$	$\frac{n}{32}$	$\frac{n}{64}$	$\frac{n}{128}$	$\frac{n}{256}$	$\frac{n}{512}$	$\frac{n}{1024}$	$\frac{n}{2048}$	$\frac{n}{4096}$	$\frac{n}{6192}$	$\frac{\text{NaOH}}{H_2O}$	H_2O
pH	3.6	3.7	3.7	3.8	4.2	4.8	5.6	6.8	7.9	8.8	9.0	9.0	7.0

FIG. 5. M/32 NaOH followed by HCl treatment. Minima at pH = 4.8 (isoelectric point). Branches of curves on left side almost identical with those of Fig. 1 (where HCl alone was applied). On the left side of isoelectric point gelatin exists as gelatin chloride, on the right side as sodium gelatinate.

teric electrolytes in general stain with acid dyes in an acid but not in an alkali solution, and with basic dyes only in an alkaline solution. This agrees with our theory that an amphoteric electrolyte like gelatin can exist as an anion capable of combining with a basic dye (*e.g.* neutral red) only on the basic side of its isoelectric point, and that gelatin can exist as a cation capable of combining with acid dyes (*e.g.* acid fuchsin) only on the acid side of its isoelectric point. Our theory further demands that gelatin when stained with neutral red should give off its dye in solutions on the acid side of its isoelectric point, since on this side of the isoelectric point gelatin can no longer exist as an anion, while gelatin stained with acid fuchsin should give off its dye in solutions with a pH >4.7, since here the gelatin can no longer exist as a cation. All this turns out as our theory demands. The writer intends to make further experiments on this problem, but the facts thus far observed support our theory that when dissociating electrolytically an amphoteric electrolyte is stable as an anion only on the basic side of its isoelectric point, and as cation on the acid side of its isoelectric point. Of course, in a non-ionized condition gelatin may exist at any pH.

Another apparent support of our theory lies in the old observation made by Hamburger[11] on the absorption of Cl and SO_4 by the red blood corpuscles from serum when the CO_2 of the blood rises. In this case it is probably simply a question of raising the pH of the blood above that of the isoelectric point for hemoglobin, which is near the point of neutrality. The whole problem of the retention and excretion of electrolytes by the body may become intelligible from the fact proved in this paper that proteins or amphoteric electrolytes in general can exist as cations only (and have a tendency to become cations) on the more acid side of the isoelectric point, while they can exist as anions only (and have a tendency to become anions) on the less acid side of their isoelectric point.

[11] Hamburger, H. J., and van Lier, G. A., *Arch. Physiol.*, 1902, 492. Hamburger, *Biochem. Z.*, 1918, lxxxvi, 309. See also, Hasselbalch, K. A., and Warburg, E. J., *ibid.*, 1918, lxxxvi, 410.

SUMMARY.

1. It has been shown in this paper that while non-ionized gelatin may exist in gelatin solutions on both sides of the isoelectric point (which lies for gelatin at a hydrogen ion concentration of $C_H = 2.10^{-5}$ or pH $= 4.7$), gelatin, when it ionizes, can only exist as an anion on the less acid side of its isoelectric point (pH > 4.7), as a cation only on the more acid side of its isoelectric point (pH < 4.7). At the isoelectric point gelatin can dissociate practically neither as anion nor as cation.

2. When gelatin has been transformed into sodium gelatinate by treating it for some time with $M/32$ NaOH, and when it is subsequently treated with HCl, the gelatin shows on the more acid side of the iso-electric point effects of the acid treatment only; while the effects of the alkali treatment disappear completely, showing that the negative gelatin ions formed by the previous treatment with alkali can no longer exist in a solution with a pH < 4.7. When gelatin is first treated with acid and afterwards with alkali on the alkaline side of the isoelectric point only the effects of the alkali treatment are noticeable.

3. On the acid side of the isoelectric point amphoteric electrolytes can only combine with the anions of neutral salts, on the less acid side of their isoelectric point only with cations; and at the isoelectric point neither with the anion nor cation of a neutral salt. This harmonizes with the statement made in the first paragraph, and the experimental results on the effect of neutral salts on gelatin published in the writer's previous papers.

4. The reason for this influence of the hydrogen ion concentration on the stability of the two forms of ionization possible for an ampho-teric electrolyte is at present unknown. We might think of the possi-bility of changes in the configuration or constitution of the gelatin molecule whereby ionized gelatin can exist only as an anion on the alkaline side and as a cation on the acid side of its isoelectric point.

5. The literature of colloid chemistry contains numerous state-ments which if true would mean that the anions of neutral salts act on gelatin on the alkaline side of the isoelectric point, e.g. the alleged effect of the Hofmeister series of anions on the swelling and osmotic pressure of common gelatin in neutral solutions, and the statement

that both ions of a neutral salt influence a protein simultaneously. The writer has shown in previous publications that these statements are contrary to fact and based on erroneous methods of work. Our present paper shows that these claims of colloid chemists are also theoretically impossible.

6. In addition to other physical properties the conductivity of gelatin previously treated with acids has been investigated and plotted, and it was found that this conductivity is a minimum in the region of the isoelectric point, thus confirming the conclusion that gelatin can apparently not exist in ionized condition at that point. The conductivity rises on either side of the isoelectric point, but not symmetrically for reasons given in the paper. It is shown that the curves for osmotic pressure, viscosity, swelling, and alcohol number run parallel to the curve of the conductivity of gelatin when the gelatin has been treated with acid, supporting the view that these physical properties are in this case mainly or exclusively a function of the degree of ionization of the gelatin or gelatin salt formed. It is pointed out, however, that certain constitutional factors, *e.g.* the valency of the ion in combination with the gelatin, may alter the physical properties of the gelatin (osmotic pressure, etc.) without apparently altering its conductivity. This point is still under investigation and will be further discussed in a following publication.

7. It is shown that the isoelectric point of an amphoteric electrolyte is not only a point where the physical properties of an ampholyte experience a sharp drop and become a minimum, but that it is also a turning point for the mode of chemical reactions of the ampholyte. It may turn out that this chemical influence of the isoelectric point upon life phenomena overshadows its physical influence.

8. These experiments suggest that the theory of amphoteric colloids is in its general features identical with the theory of inorganic hydroxides (*e.g.* aluminum hydroxide), whose behavior is adequately understood on the basis of the laws of general chemistry.

Addendum. Since this paper went to print the writer has been able to show by volumetric analysis that the view expressed in this paper is correct; namely, that on the alkaline side from the isoelectric point gelatin combines with the cation of a neutral salt,

while on the more acid side from the isoelectric point it combines with the anion of a neutral salt.   The curve for the amount of cation or anion in combination with the gelatin runs parallel with the curves for osmotic pressure, swelling, etc.   The new experiments will be discussed in the second number of this *Journal*.

A THEORY OF THE MECHANISM OF DISINFECTION, HEMOLYSIS, AND SIMILAR PROCESSES.

By S. C. BROOKS.

(From the Harvard School of Tropical Medicine, Boston.)

The apparent course of such processes as hemolysis is determined by the rate of change in the number of living cells which have undergone some definite alteration, such as laking or loss of viability. A great deal of confusion has arisen from the attempts of various investigators to deduce from the observed course of disinfection and hemolysis the nature of their fundamental reactions. Processes of this kind have often been looked upon as due to reactions of a monomolecular type, solely because of a superficial resemblance between the curves expressing the rate of progress of the two phenomena.

This paper is a critical discussion of the part played by the physico-chemical process or group of processes leading to death, laking, and similar effects in determining progressive changes in the number of individual cells succumbing in successive units of time to the action of the deleterious agent. These physicochemical processes in the protoplasm may, for the sake of brevity, be termed the "fundamental reaction;" by the "course of the process" we shall understand the time curve of any process like hemolysis or disinfection. The subject is treated in four sections dealing with (1) the evidence that the degree of hemolysis is determined by the number of cells which are laked, and that this depends on the fact that individual cells possess different degrees of resistance; (2) the influence taken singly and in combination, of changes in the variation curve of resistance and of progressive changes in the velocity of the fundamental reaction; (3) the hypotheses advanced to account for the observed course of disinfection, hemolysis, and like processes; (4) the interpretation and significance of the time curves of such processes.

I.

If erythrocytes are suspended in an indifferent medium and subjected to a brief radiation from a mercury vapor arc in quartz, or if they are suspended in an appropriate dilution of serum acting in conjunction with a specific antibody, there ensues a gradual liberation of hemoglobin. In both cases this process begins at a rather slow rate which gradually increases, passes through a maximum, and then gradually falls off until it becomes comparable with the rate of spontaneous laking. (See Table I.)

TABLE I.

Observed Course of Hemolysis; 100 Per Cent Signifies Completion.

10 min. exposure to Hg. arc in quartz.		Specific serum hemolysis.						
		Experiment 26, complement 0.2 per cent.			Experiment 20, complement 0.67 per cent.		Experiment 20, complement 0.23 per cent.	
Time after radiation.	Colorimetric estimation.	Time.	Color.	Cell count.	Time	Color.	Time.	Color.
min.	*per cent*	*min.*	*per cent*	*per cent*	*min.*	*per cent*	*min.*	*per cent*
5	1	0	0	0	0	0	0	0
12	2	3	3	—	10	65	30	5
19	3	6	5	12	20	94	60	28
37	5	10	10	—	40	100	120	38
55	12	14	21	45			210	45
88	29	20	30	—				
145	55	28	56	55				
205	72	41	67	73				
295	87	56	74	—				
660	100	85	81	79				
		112	81	—				
		155	82	82				
		1,320	100 .	100				

Plotting as ordinates the amount of hemoglobin liberated, and as abscissæ the times of sampling, one obtains asymmetrical sigmoid curves such as those shown in Fig. 1. The gradual retardation and final apparent equilibrium is either due to exhaustion or inactivation of the lytic agent, or if the concentration of the lytic agent (photoproduct or serum) is increased above that necessary to produce complete hemolysis in a few hours, the process ceases because of the ex-

haustion of the supply of cells.   (In the latter case we may conceive
that the fundamental reaction is still proceeding at a relatively rapid
rate even in the most resistant cells at the time when they succumb.)

Enumeration of the red blood cells visible in a given volume of sus-
pension at various stages of hemolysis shows that the amount of
hemoglobin liberated is very nearly proportional to the number of
cells which have lost their pigment (Table I and Fig. 1).   Handovsky[1]
finds a similar parallelism between the disappearance of cells and the
liberation of hemoglobin when erythrocytes are partially hemolyzed
by saponin in low concentrations.
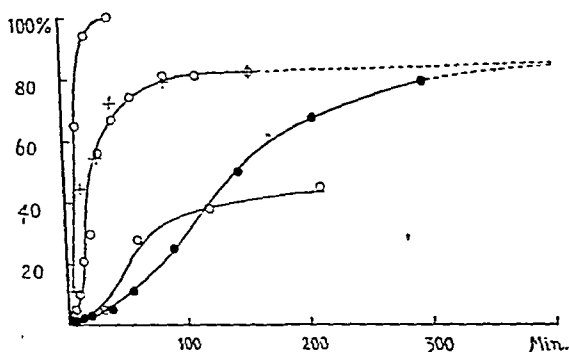
FIG. 1. The time curve of hemolysis produced by ultra-violet radiation (solid
circles), and by various dilutions of complement acting on specifically sensitized
cells (open circles): the degree of hemolysis was colorimetrically determined.
The crosses represent the degree of hemolysis as determined by cell counts during
the course of the process represented by the adjoining curve.   The ordinates
represent the amount of hemolysis in per cent of completion, and the abscissæ,
time in minutes.

The course of hemolysis depends therefore on the relative number of
red blood cells having in different degrees the power to resist the ac-
tion of the lytic agent. For since the amount of hemoglobin liberated
is always proportional to the number of erythrocytes which have dis-
appeared we must regard the observed course of hemolysis as the
summation of the laking of individual cells at varying times after they
are subjected to the condition leading to hemolysis.   A few relatively

[1] Handovsky, H., Arch. exp. Path. u. Pharmakol., 1912, lxix. 412.

fragile cells are laked almost immediately, the resistant ones survive for many hours, but most of the cells succumb during an intermediate period when the observed rate of hemolysis is 'at a maximum. When hemoglobin finally begins to diffuse from a given erythrocyte, the process is so quickly completed that it may ordinarily be regarded as instantaneous. It is obvious that an increase in the relative number of, for example, the more fragile cells would accelerate the earlier stages of hemoglobin liberation, while a decrease would produce a corresponding retardation.

II.

There are two ways of expressing graphically the progress of hemolysis: the time curve or course, whose ordinates are proportional to the total number of cells laked; and, the rate curve, whose ordinates are proportional to the number of individuals laking in a unit of time. The former is the more frequently found in hemolytic studies, while the latter is the "mortality curve" of vital statistics. If one of these curves is known the other may be found by graphical methods. For instance, let us suppose that we have only the mortality curve: the ordinates of the time curve represent the total number of individuals having insufficient resistance to survive beyond the indicated time; the area enclosed by the mortality curve corresponds to the total number of individuals; the area enclosed by the ordinate at any point on the x axis and the part of the mortality curve to the left of the ordinate, is proportional to the number of cells having less than the indicated resistance, and is therefore also proportional to the corresponding ordinate of the time curve. If the mortality curve is plotted on suitable coordinate paper these areas may easily be estimated with sufficient accuracy, ordinates proportional to them erected at the corresponding points on the x axis, and their tops connected by a smoothed curve. This will be the time curve, and, since the process of finding it is a process of integration, it is at the same time an integral curve.

If we have only the time curve, or integral, it is easy to see that the rate of hemolysis, that is the number of cells laking in a unit of time, is represented by the slope of the time curve; the steeper the time

curve, the more individuals are succumbing per minute. The ordinates of the mortality curve are then at any time proportional to the steepness of the time curve. The slope or steepness is best found by plotting the time curve on suitable coordinate paper, drawing straight lines tangent to it at several points, and counting the number of squares passed over vertically by such lines for a given number passed over horizontally. This ratio is the trigonometric tangent of the slope of the time curve. If we draw ordinates proportional to these ratios at corresponding points on the x axis, and connect the tops of these ordinates by a smooth curve, we shall obtain the rate or mortality curve. Since the process by which we have gotten the rate curve is a graphic method of differentiation, we may consider the curve to be the differential of the time or integral curve.[2]

Under certain conditions the mortality curve may be identical with the variation or frequency curve of individual resistance. In the following paragraphs the relationship between the time curve and the variation curve is considered, starting with the simplest imaginable conditions, then varying the frequency curve alone, the course of the fundamental reaction alone, and finally both together.

If the rate of hemolysis is uniform, its time curve would be a sloping straight line (the integral curve, a, Fig. 2), while since its tangent or slope is the same at every point, the differential or mortality curve would be a straight line parallel to the axis of the abscissæ (b, Fig. 2); this condition could be expressed by the differential equation

$$\frac{dn}{dt} = k \qquad \qquad '$$

where n is the number of cells, t the time, and k is a constant.

If we now assume the cells to be divided into classes differing from each other by one unit of resistance (r) (defining as a unit of resistance the power to resist one unit of fundamental reaction; e.g., the formation of one mol of toxic substance), and further assume that the fundamental reaction is proceeding at a uniform rate, the differential equation may be replaced by the equation of a frequency curve, or

[2] This relationship has been suggested by Davey (J. Exp. Zöol., 1917, xxii, 573), in connection with curves representing the per cent of death occurring among flour beetles (Tribolium confusum) at various times after X-radiation.

variation curve in which all classes are equal in respect to number of individuals. This equation is $y = y_0$, where y_0 is the number of individuals in any one of the equal classes which is arbitrarily selected as the mode, and y is the number of individuals having x units of resistance less or more than the mode. It is obvious that in this particular case any class may be the mode. When the equation assumes a different form so that the classes are unequal (as in the ordinary curves found in biological work) and in consequence y varies, y_0 is the maximum ordinate of the variation curve.
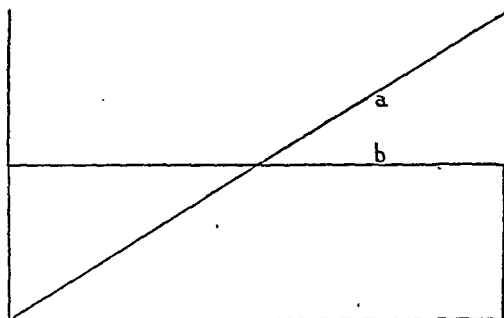
FIG. 2. The relation between the time curve (a) of a reaction proceeding at a constant rate, and the curve of the differential equation (b). The ordinates represent the extent to which the reaction has proceeded (a) or the rate at which it is proceeding (b), and the abscissæ represent time.

In order to understand the effect of changes in the shape of such variation curves, that is of changes in the relative number of cells having different degrees of resistance, let us retain the assumption that the fundamental reaction proceeds at a uniform rate; for this allows us to think of the variation curve (abscissæ = resistance) as being at the same time the differential of the time curve (abscissæ = time). Now suppose the variation curve to have the form (a, Fig. 3) commonly found in biological statistics, of a "skew frequency curve of limited range" whose equation according to Pearson[3] is

$$y = y_0 \left(1 + \frac{x}{x_1}\right)^{k x_1} \left(1 - \frac{x}{x_2}\right)^{k x_2} \qquad (1)$$

where k is a constant, and x_1 and x_2 the number of degrees of resist-

[3] Pearson, K., *Phil. Tr.*, A, 1895, clxxxvi, 343.

ance less and more, respectively, than the mode, possessed by the most fragile and most resistant classes. Here $x_1 + x_2$ is the total range of resistance of all the cells. A curve of this type (a) and its integral, or time curve, (b) are shown in Fig. 3; the time curve has a form not unlike that of the curves for the course of hemolysis.

' If we suppose, with some investigators, that the resistance of the cells varies around an average from which it does not deviate to an extent sufficient to influence the course of the process, we must consider all the cells to be in a single class with respect to resistance.
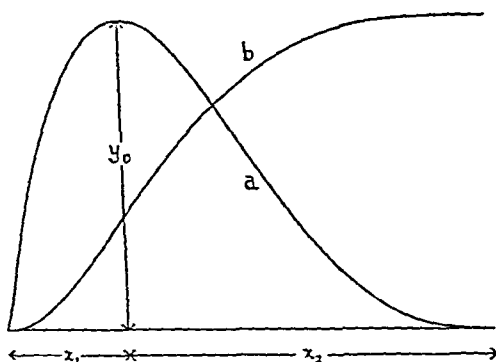
Fig. 3. (a) variation curve whose equation is

$$y = y_0 \left(1 + \frac{x}{x_1}\right)^{kx_1} \left(1 - \frac{x}{x_2}\right)^{kx_2}, \text{ where } x_1 = 2, x_2 = 6, k = 0.5.$$

If (a) is considered as the curve of a differential equation, (b) is the curve of the corresponding integral. The ordinates of (b) are proportionate at any position on the x axis (abscissæ) to the area to the left of the ordinate at that position, and under the curve (a).

The frequency curve will then be so narrow as to be approximately a straight line normal to the axis of the abscissæ at some point. Its integral, which is the time curve of the process, will follow the axis of abscissæ to this point, and then rise perpendicularly to its ultimate height. In other words, if the process were hemolysis, all the cells would lake at the same instant. This conclusion may be avoided, theoretically at least, by making one of the special assumptions which are discussed below.

As a matter of fact the exact shape of the frequency curve—that is the relative abundance of cells having different degrees of resistance to any given lytic agent—is not of general significance, for it depends on the condition and previous treatment of the animal from which the cells are secured, on the nature of the lysin, etc. For example, Handovsky[1] has shown that during the regeneration of erythrocytes following artifically induced anemia there are two groups of erythrocytes, one of which has a higher average resistance to hemolysis by saponin than the other; differentiation of curves representing the course of the hemolysis of such blood cells gives a bimodal variation curve. The same author has shown that alterations in resistance may appear in opposite senses according to the choice of lytic agents; *e.g.*, the blood cells of dormant bats are less resistant to the action of saponin, but more resistant to that of sodium hydroxide than those of active bats.

We may sum up the influence of variations of resistance by saying that they determine the general shape and points of inflection of the curves expressing the course of such processes, and that, therefore, in the absence of further analysis such curves tell us nothing as to the nature of the fundamental reaction.

Changes in velocity during successive stages of the fundamental reaction will obviously produce corresponding changes in the time required to produce a given degree of hemolysis, and will therefore alter the shape of the time curve. We have seen that when the equation of the variation curve of resistance is of the form

$$y = y_0$$

and the velocity of the fundamental reaction is constant, the time curve of the reaction is identical with that of the process as a whole. A moment's reflection will show that, whatever the course of the fundamental reaction, so long as the variation curve remains of this form the same identity will appear. If for example the fundamental reaction is monomolecular, the course of the process will appear monomolecular.

The relative length of time required for the process to reach any given stage (*i.e.* the abscissa of the time curve for any given ordinate) will be greater for the same initial velocity in the case of a monomo-

lecular reaction than in the case of a reaction proceeding at a constant rate, and the lengths will be still greater for reactions of higher orders.

The relative times, l_1 and l_0, required for two reactions with the same initial velocity to reach the same stage, when one is monomolecular and the other has a constant velocity, may be easily derived from the equations of the two time curves. The expression is

$$\frac{l_1}{l_0} = \frac{1}{x} \ln \frac{a}{a-x}$$

where ln is the logarithm to the base c ($2.3026 \log_{10} \frac{a}{a-x}$ may advantageously be used in place of $ln \frac{a}{a-x}$). and where a is the initial amount of the reacting substance, and x the amount transformed at any given stage; a and x are usually stated in per cent.

If we compare the rates of hemolysis which would result from the action of two fundamental reactions of different orders. we find that the relative time required to reach a given stage will be the same regardless of the variation curve of the cells affected; if the shape of the variation curve is given by some equation other than $y = y_0$ (which gives identical time curves for the fundamental reaction and for the process as a whole), we can still calculate the relative lengths of time required for the process to reach a given stage, according to the order of the fundamental reaction. If the fundamental reaction is monomolecular or of a higher order. we must use as abscissæ distances which are as much greater than those of the time curve when the fundamental reaction has a constant velocity, as is indicated by calculations like that given above. This is most easily done if it is assumed that the hemolysis and the fundamental reaction reach completion simultaneously, but may still be calculated for a known excess or deficiency of the lytic agent. In Fig. 4 are given the courses which would be assumed by a process taking place in a group of cells, the equation of whose variation curve was

$$y = y_0 \left(1 - \frac{x^2}{x_1^2} \right)^{k x_1}$$

if the fundamental reaction were (a) proceeding with a constant velocity; (b) proceeding according to the law of monomolecular

reactions.   Here again we find a curve *b* which simulates the observed curves for the course of hemolysis.   We shall return to this point later on.
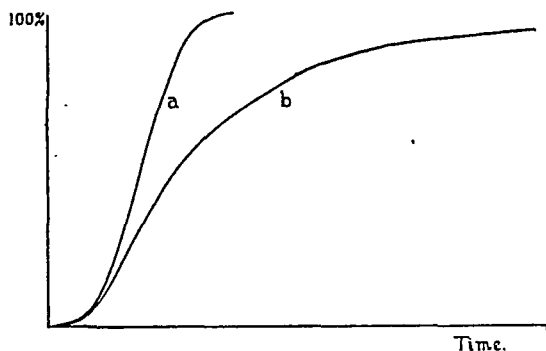
FIG. 4. The time curve of a process occurring in a group of individuals having a variation curve with the equation $y = y_0 \left(1 - \dfrac{a^2}{x_1^2}\right)^{kx_1}$, if the fundamental reaction proceeds at a uniform velocity (a), and if it proceeds according to the law of monomolecular reactions (b).   Here $x_1 = 1.5$ and $k = 2$.   The ordinates represent degree of completion of the process, and the abscissæ, time.

III.

Various investigators have reported that bacteria in the presence of disinfectants die at a rate which is at any given time proportional to the number of organisms then surviving.[4-9]   This condition would be described by the equation

$$\frac{dn}{dt} = kn_t, \text{ or } n_t = n_0 e^{-kt}$$

where n_0 and n_t are the number of bacteria at the beginning of the experiment and at t units of time thereafter, and e is the base of the natural system of logarithms.   This is the equation of the mono-

[4] Madsen, T., and Nyman, M., *Z. Hyg.*, 1907, lvii, 388.

[5] Chick, H., *J. Hyg.*, 1908, viii, 92.

[6] Chick, *J. Hyg.*, 1910, x, 237.

[7] Phelps, E. B., *J. Infect. Dis.*, 1911, viii, 27.

[8] Eijkman, C., *Folia Microbiol.*, 1912, i, 359.

[9] Reichenbach, H., *Z. Hyg.*, 1911, lxix, 171.

molecular reaction isotherm. Similar relationships have been reported in the case of hemolysis, which is a process in many ways similar to disinfection;[10-14] although in this case a so called "induction period," often of considerable length (Dreyer and his coworkers) intervenes before the hemolysis appears to follow the course of a monomolecular reaction. In both hemolysis and disinfection a large number of single living cells are exposed to the action of an agent which ultimately induces in the cell some change which we can detect; in one case loss of power to reproduce; in the other, loss of a pigment; in both cases there is great variation in the length of time required to bring about the critical change in different cells. Harvey[15] considers the equation for monomolecular reactions to be applicable to the loss of motility suffered by cells of *Chlamydomonas* subjected to the action of hydrochloric acid in great dilution; while Darwin and Blackman, according to Arrhenius,[16] saw the same relationship when they allowed various killing agents to act on seeds. These citations, while by no means complete, will suffice to indicate the wide range of phenomena which have been studied from this point of view.

Some authors, like Dreyer and his coworkers, have not attempted to attribute the apparent analogy of these processes with monomolecular reactions to any single relationship, but some other authors have devoted to this phenomenon a great deal of discussion which does not seem to have been based upon a comprehensive knowledge of the subject, for their attempts to explain the analogy will not bear criticism, and none of their criticisms are entirely satisfactory. The difficulty is largely due to failure to see the necessary consequences of uniformity in resistance, or to disregard of the possible influence of progressive changes in the velocity of the fundamental reaction.

[10] Arrhenius, S., and Madsen, T., *Z. physik. Chem.*, 1903, xliv, 33.

[11] Henri, V., *Compt. rend. Soc. biol.*, 1905, lvii, 37.

[12] von Liebermann, L., and von Fenyvessy, B., *Z. Immunitätsforsch., Orig.*, 1912, xii, 417.

[13] Salomonsen, C. J., and Dreyer, G., *Compt. rend. Acad.*, 1907, cxliv, 999.

[14] Dreyer, G., and Hanssen, O., *Compt. rend. Acad.*, 1907, cxlv, 371.

[15] Harvey, H. W., *Ann. Bot.*, 1909, xxiii, 181.

[16] Arrhenius, S., Quantitative laws in biological chemistry, London, 1915, interprets in this way the experiments of Darwin, N., and Blackman, A., *Rep. 78th Meeting, 1908, Brit. Assn. Adv. Sc.*, 1909, 902:

Madsen and Nyman,[4] who were the first to notice the analogy, recognized the fact that variability among the cells was a factor to be reckoned with, but appear nevertheless to have regarded their curves as expressions of the average rate of change in the individual cells.   We have seen that it is impossible, without special assumptions, to account for the phenomenon on this basis.   Miss Chick,[5] working independently, secured data like those of Madsen and Nyman, but states explicitly that the monomolecular reaction formula is applicable to the process, "one reagent being represented by the disinfectant" which being present in excess may be regarded as having a constant concentration, "and the second by the protoplasm of the bacterium;" she amplifies this statement by making the supposition that the bacteria undergo rapid cyclic variations in their ability to react with the disinfectant.   Phelps[7] in developing Miss Chick's method for standardizing disinfectants, adopts the same explanation. Arrhenius[16] says, "there is no doubt that the different cells in a sample of bacteria or red blood-corpuscles possess a different power of resistance to deleterious substances," but that "the different lifetime of the different bacteria does not, therefore, depend in a sensible degree on their different ability to resist the destructive action of the poison," and accepts Chick's explanation, as does Eijkman,[8] at least in the case of certain bacteria.

The acceptance of such an explanation makes it necessary to assume that loss of viability, like the breaking up of a single molecule of saccharose during inversion, takes place in a single step; in other words, that the disinfectant cannot have any cumulative effect on the viability of individual cells.   If the loss of viability occurred in two or more steps, some or all of the cells surviving at any time during the process would be "partially dead," and a greater proportion of them would succumb in any given interval of time than would have done so during the same interval at the beginning of the process when all of the cells were entirely unaffected.   In other words the per cent death would increase during the process instead of remaining constant as demanded by the law of monomolecular reactions.

This assumption that death occurs at a bound, as it were, is surprisingly at variance with the usual conception of vital processes.   It seems to necessitate that we regard a living cell as being dynamically

comparable to a molecule. For this reason Robertson[17] has offered another explanation for the apparent exponential decrease in the number of surviving cells; *i.e.*, for the apparent applicability of the law of monomolecular reactions. His explanation assumes the collisions with the disinfectant molecules to be distributed fortuitously among the different individuals of a homogeneous group of cells. Since it would be out of place to consider here the details of his mathematical proof, it must suffice to point out that Robertson's quantity x, "the number of units of the underlying change," must apparently be at one and the same time a constant, and an exponential function of time. This impossible assumption is the basis of Robertson's whole proof. Other mathematical inconsistencies occur, but are of relatively little consequence.

In a subsequent paper[6] Miss Chick modifies her original theory by assuming that it is the protein molecules of the bacterial protoplasm, which, like the sugar molecules during hydrolysis, undergo the cyclic changes in energy content, upon which depend their ability to react. The concentration of these protein molecules at any moment would then, according to Miss Chick, determine the rate of death of the bacteria at that moment. We have already seen that under these conditions the cells of a group of uniformly resistant individuals, such as Miss Chick postulates, would all die at one time. If the course of disinfection is to parallel that of the reaction, the diametrically opposite condition must prevail; namely, one in which the cells are equally distributed among all the possible classes of resistance; in other words, the equation of the variation curve must be $y = y_0$.

Von Liebermann and von Fenyvessy[12] explained the hemolytic "monomolecular curve" on the basis of the probable rate of exit of hemoglobin from the individual cell, from which the pigment might be supposed to diffuse at a rate proportional to the difference between the intra- and extracellular hemoglobin concentrations. This explanation is evidently in conflict with the observed progressive decrease in the number of intact erythrocytes, and with the fact reported by Dienes[13] that there is a nearly constant ratio between the hemoglobin

[17] Robertson, T. B., *J. Hyg.*, 1914, xiv, 143.
[13] Dienes, L., *Biochem. Z.*, 1911, xxxiii, 268.

content and the dry weight of the residual cells at various stages of hemolysis.

Since none of the explanations given above is satisfactory let us see whether the experimental facts necessitate the assumption that the equation of the monomolecular reaction is a complete description of the process of disinfection. Examination shows that none of the experiments already referred to include the observations necessary for the study of the first part of the process; the second observation was almost without exception deferred until the process was nearly half complete. It also appears that in many experiments the "initial" number of cells was determined after an appreciable part of the process had occurred, that is after the most fragile cells had already succumbed, and while the reaction was proceeding so rapidly that the slightest errors in the time of sampling would produce great differences in the number of cells recorded.

It is quite impossible to judge correctly the validity of formulas based on such inadequate data. In Fig. 5, I have plotted the points of that one of Chick's curves which shows the best agreement with a monomolecular reaction curve, a similar curve for the number of *Chlamydomonas* cells immobilized at intervals after exposure to dilute hydrochloric acid (Harvey,[15]), a curve for normal serum hemolysis (Henri,[11]), and an original curve (from which the first few observations are omitted) for hemolysis after ultra-violet radiation. In the last two cases hemolysis was estimated colorimetrically. A monomolecular reaction curve is drawn for comparison with these points, and the scales of the abscissæ are so arranged as to make the curves coincide at the point where the effect is half completed. The agreement of the points with the monomolecular reaction curve is about equally close. But in this same figure the curve for the original hemolytic experiment is also drawn with the abscissæ on a larger scale and with the first few observations included. It is obvious that these first few observations are most important. One suspects that had these observers but made sufficient observations during the first part of the process, they would have found that disinfection, killing of seeds, etc., are processes which, like hemolysis, are initially slow and only subsequently attain their maximum velocity. As a matter of fact this is what a number of other workers have found.

Dreyer and his coworkers, for example, found that only after a considerable period of slow laking, did the course of the hemolysis induced by α and β rays of radium and by ultra-violet radiation follow the course of a monomolecular reaction; this initial lag they call, tentatively, a period of induction.

Eijkman[8] has shown that the shape of the disinfection time curve is peculiar to the particular culture of bacteria used, and to some
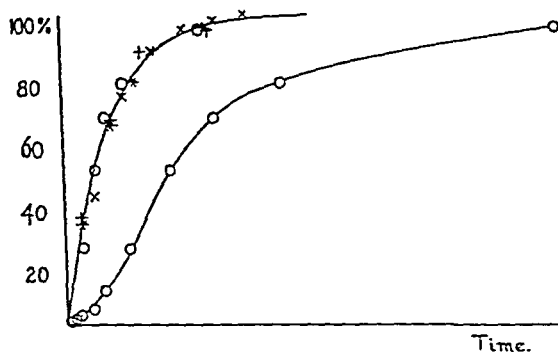
FIG. 5. The upper curve is the monomolecular reaction isotherm, beside which are plotted experimentally determined points for normal serum hemolysis (Henri, asterisks), the immobilization of *Chlamydomonas* by 0.009 per cent HCl (Harvey, diagonal crosses), disinfection (Chick. erect crosses), and hemolysis by ultra-violet radiation (original, circles); the abscissæ were so adjusted in plotting these points as to make their curves coincide with the monomolecular reaction isotherm at 50 per cent. The lower curve represents the course of the same ultra-violet hemolysis, but with the abscissæ plotted on a larger scale, and with the addition of 5 points determined during the first part of the process. The ordinates represent degree of completion of the process, and abscissæ, time.

extent characteristic of the species. Both Eijkman and Reichenbach[9] have secured disinfection time curves which, even when determinations during the early stages of the process are included, do not diverge very greatly from the exponential or monomolecular type of curve. It is possible to explain such curves either by assuming a monomolecular fundamental reaction and a variation curve of the form $y = y_0$, or by assuming a fundamental reaction proceeding at a uniform rate, and a variation curve of the form

$$y = y_0 e^{-kx} \tag{2}$$

where k is a constant. The latter equation is that of the monomolecular reaction isotherm; but it gives a curve having unlimited range, a condition which makes it undesirable in the case of a limited population; it is better to use a curve of very similar appearance which results if we put $x_1 = 0$ in equation (1) above. We thus introduce the necessary factor of limited range given by x_2. The equation, so modified, becomes

$$y = y_0 \left(1 - \frac{x}{x_2}\right)^{kx_2} ,$$

while if $\frac{x}{x_2}$ is made indefinitely great (*i.e.* if we consider only the first part of a curve with infinite range) it approaches an equation identical with (2) above.

Reichenbach has shown that if we imagine a constant fraction of the bacteria of each generation to lose their power to divide, and suppose that at each successive division there occurs a decrease in the resistance of the dividing organisms, the individuals with the highest resistance, *i.e.* those of the first generation, which have not since undergone division, will be present in the smallest numbers, and that those of succeeding generations (and which have therefore less resistance), will be present in numbers increasing in geometrical proportion. Under certain conditions such a culture would have nearly the type of variation curve necessary to give the "monomolecular" curve of killing; and Reichenbach finds in individual variation a complete explanation of the observed curves of disinfection. This hypothesis is not, however, applicable to the process of hemolysis, and can therefore have no general significance. The agreement of the middle portion of hemolytic and other similar time curves with the monomolecular reaction curve is probably only a coincidence, and is not of fundamental significance.

Yule[19] has shown that it is possible, theoretically at least, to account for a time curve of hemolysis such as that which I have observed, which, rather than a monomolecular curve, is probably characteristic of the other processes here discussed, even if all the cells are assumed to be equally resistant. This involves the assumption that a certain small number, r, of independent changes suffices to cause lysis. Probably in the case of hemolysis r would not greatly exceed 2. If $r = 1$

[19] Yule, G. U., *J. Roy. Statist. Soc.*, 1910, lxxiii, 26.

Yule's formulas lead to a "monomolecular" curve. It seems more rational to adopt some explanation which takes into account the individual variations in resistance, thus avoiding unnatural assumptions as to the nature of the fundamental reaction.

IV.

The idea that the rate of disinfection is due to variations in resistance is not new; Geppert[20] gave expression to it nearly a generation ago. The same idea has since been restated in many forms and in many connections, but always without reference to any influence which might be exerted by the fundamental reaction.

Mioni [21,22] criticizes Henri's studies[11] on hemolysis, because Henri neglected the influence of individual variation; Mioni's experimental evidence is inconclusive, however. Dienes[18] suggested that various degrees of resistance to hemolysis were distributed in accordance with "Quetelet's law" $(y = y_0 e^{-kx})$. Hewlett[23] says, with reference to the hypothesis offered by Miss Chick in her first paper, "While admitting that the disinfection of anthrax spores follows the *course* of a unimolecular reaction, I think it extremely doubtful, to say the least, if the reaction between disinfectant and bacterium *is* a unimolecular reaction." Hewlett's own experiments on the effect of mercuric chloride on the viability of mustard seeds accord with those of Darwin and Blackman,[16] but he says: "It appears to me that only by a wide stretch of the imagination can the interaction of mustard seed and disinfectant be considered as a unimolecular reaction, or a reaction of a higher order, yet it follows approximately the course of the former."

Loeb and Northrop[24] also criticize the idea that disinfection is due to a monomolecular reaction. They say Miss Chick "was probably led to such an assumption by the fact that the ascending branch of the mortality curve in her experiments was generally very steep . . . . almost a vertical line." thus escaping detection. Hence she noticed usually only the less steep descending

[20] Geppert, J., *Berl. klin. Woch.*, 1889, xxvi, 789.

[21] Mioni, G., *Ann. Inst. Pasteur*, 1905, xix, 84.

[22] Mioni, *Compt. rend. Soc. biol.*, 1905, lvii, 485.

[23] Hewlett, R. T., *Lancet*, 1909, i, 889.

[24] Loeb, J., and Northrop, J. H., *J. Biol. Chem.*, 1917, xxxii, 103.

branch which could be interpreted as a monomolecular curve for the reason that her experiments lasted only a short time." Miss Chick's published curves were, as a matter of fact, time curves, *i.e.* "integrals" of "mortality" or variation curves, and had she been able to make sufficient observations at the beginning of the process, she might have obtained curves at first horizontal, but very quickly turning downwards. Such a condition would find expression in a mortality curve as an ascending branch rising very steeply from zero to a maximum.

The true explanation of the course of processes like disinfection is undoubtedly a combination of the two extreme views: one attributing the course to variation alone, the other considering it to express the nature of the fundamental reaction alone. Both of these factors exert an extremely important influence. We have seen that it is theoretically possible to relegate variation to a position of unimportance by assuming a variation curve having the form $y = y_0$. Such a distribution of various degrees of resistance is obviously unnatural, and the assumption that it occurs must, in the absence of definite evidence, be considered unwarranted. Its use frequently necessitates the postulation of "latent" or "induction" periods of whose existence we have no further proof. On the other hand we may consider the reaction velocity as constant, and by graphic differentiation obtain not unnatural frequency curves. Perhaps by employing several constants we may even obtain applicable equations; but the constants will have no physical meaning, and the equations no general significance.

The reaction velocity, as a moment's consideration will show, must ordinarily decrease as the process goes on; for "sub-minimal" amounts of the toxic substances carry the process to partial completion, and whatever the final "equilibrium" may be, it is gradually attained; the process does not abruptly cease.[25]

[25] Curves drawn through points expressing the amount of toxic agent required to produce various degrees of completion of such processes might be regarded as "integrals" of the frequency curves of resistance. In practice, however, this reasoning is applicable only to such *in vivo* experiments as determination of therapeutic efficiency or toxicity of drugs, radiation, etc. *In vitro*, factors such as bacterial contamination, autolysis, cell division, or starvation are likely to supervene, and distort the observed curve.

Should we not then conclude that the course of processes like disinfection is, like *b* in Fig. 4, the result of the simultaneous operation of two factors: the frequency curve of variation in individual resistance, which may be different for each group of cells and each toxic agent; and the course of the fundamental reaction, which usually proceeds with a velocity diminishing during the experiment at a rate dependent on the particular conditions prevailing? We must also bear in mind that what we have supposed to be the fundamental reaction may be the end result of a complex series of interrelated or "catenary" reactions. If some one link in this chain of events is a change which, from the beginning to the end of the process, is so slow as to govern the rate of the whole series, which taken together is regarded as the fundamental reaction, then, and only then, will orderly laws describe the course of the latter.

This conception does indeed render a solution of the problem much more difficult than it once seemed, but not necessarily unattainable. A proper understanding of this "group experiment," as we might perhaps call the widely employed type of which disinfection is but one example, should lead to better interpretation of the phenomena themselves, and a far deeper insight into the fundamental life processes to which they are due.

CONCLUSIONS.

1. The course of such processes as hemolysis is very largely dependent upon variations in resistance among the different individuals, and secondarily upon the course of the fundamental reaction.

2. The fundamental reaction may be either a simple process, or the expression of a complex series of changes whose rate is at all times governed by that of the slowest of the series. This might perhaps be regarded as another expression of the so called "Law of the minimum."

3. Unnatural assumptions would be requisite for the explanation of a resemblance between the course of such processes in general and that of a monomolecular reaction.

4. The supposition that such a general resemblance exists is not supported by the available evidence.

5. The independent determination of either the nature of the fundamental reaction, or the type of the variation curve for the particular case under observation, will further our knowledge of the nature of such processes and lead to a far deeper insight into the nature and reactions of living matter.

THE LAW CONTROLLING THE QUANTITY OF REGENERA-
TION IN THE STEM OF BRYOPHYLLUM CALYCINUM.

By JACQUES LOEB.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

I.

It is well known that isolated pieces of plants and lower animals are able to replace the lost parts by a new growth which is called regeneration. The investigation of this phenomenon by merely qualitative methods of experimentation has led only to verbalisms. Thus it has been stated by Noll that the plant or animal possesses a "morphestesia;" *i.e.*, a consciousness of what its proper form should be and hence the regeneration of lost organs. Driesch applies a similar verbalism calling the morphestesia "entelechy." Weismann suggests that without the power of regeneration many species might have died out—hence regeneration. Others speak of regeneration as the effect of the "stimulus" of the wound. If we wish to substitute for these or similar expressions, which have led and can lead to no scientific result, a theory in the sense in which this word is used by the physicist, we must investigate the problem of regeneration by the methods of quantitative experimentation.

The writer has introduced such a method in the study of regeneration of the plant *Bryophyllum calycinum*, which is unusually favorable for work of this kind, and he has already reported some of the results obtained. The most significant fact was found in the regeneration of the leaf of this plant. When the leaf of *Bryophyllum* is detached from the plant, it will form roots and shoots in its notches. Each node of the stem has two leaves in opposite position—sister leaves —which under normal conditions have equal size. Since they have also the same age and the same history, they possess not only the same mass but contain also very probably chloroplasts in the same number and in the same degree of efficiency; so we can say such sister leaves

have approximately equal masses of *active* substance.   The writer was able to show *that such sister leaves of equal weight produce equal masses of shoots in equal times and under equal conditions of illumination, temperature, and moisture.*   He found, moreover, that if we reduce the mass of one set of sister leaves by cutting out pieces from the center of the leaves, while the other set remains intact, both sets produce shoots approximately in proportion to their masses, even if the number of shoots produced by the two sets differs widely.   We, therefore, can say that *equal masses of sister leaves produce equal 'masses of shoots in equal times and under equal conditions, regardless of the number of shoots produced.*[1]

This law shows that the problem of regeneration is part of the problem of growth and that it falls under the law of chemical mass action.

Inasmuch as age and previous history influence the active mass of photosynthetic material of the leaf, it is obvious that the law of the production of equal masses of shoots by equal masses of leaves is fulfilled more accurately by comparing sister leaves than it would be by comparing leaves with a different history; *i.e.*, leaves which differ in the mass of photosynthetic material in the unit mass of leaf.   In such cases we should have to use statistical methods; *i.e.*, we should be compelled to use much larger numbers of leaves in order to eliminate the influence of variation in the relative mass of photosynthetic and other material in the leaf which is required for the regeneration (growth) of the shoots.

II.

Regeneration in an isolated piece of stem is much more general than regeneration in an isolated leaf, and the question arises whether a similar mass law, as that found for regeneration in a leaf, controls the quantity of regeneration in a stem.

In the axil of each leaf of the stem of *Bryophyllum* is found a dormant bud capable of growing into a shoot (3, Fig. 1).   Each node contains two such dormant buds on opposite sides, one in the axil of each leaf.   In successive nodes the lines connecting the two leaves

---

[1] Loeb, J., *Science*, 1917, xlv, 436; *Bot. Gaz.*, 1918, lxv, 150; *Ann. Inst. Pasteur*, 1918, xxxii, 1.

and their axillary buds are at right angles to each other.   If we, there-
fore, split a stem longitudinally into two halves (Fig. 1) in such a way
that the plane of section is at right angles to the line connecting the
two most apical buds (1. Fig. 1), the section cuts through the buds in
the next node (2, Fig. 1), and injures them more or less, while it leaves
the buds of the second next (3, Fig. 1) node intact, and so on.   It
happens for reasons which need not be discussed here that in such a
stem as a rule only the most apical bud (1. Fig. 1) grows out.
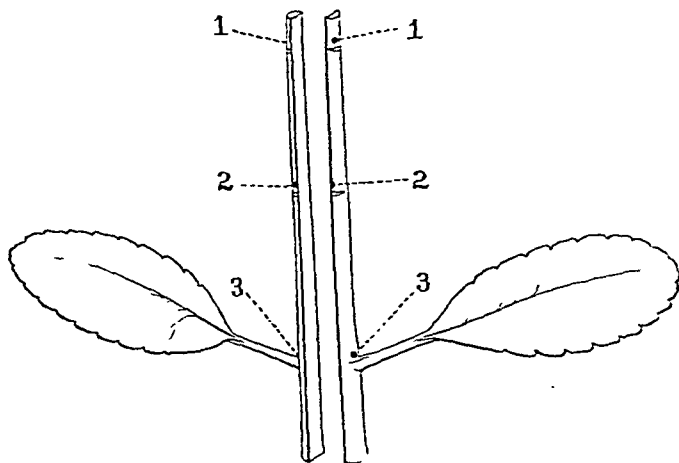
Fig. 1. Diagram showing the method of splitting the stem longitudinally
in order to investigate the influence of mass of basal leaf upon shoot production.
1, 2, 3, designate the three pairs of dormant buds, two in each of the three nodes of
the stem.

The following method was used for testing whether or not the
quantity of regeneration in a piece of stem is controlled by the simple
mass law which holds for the regeneration of shoots in the stem.
From the stem of *Bryophyllum* were cut pieces containing three nodes
(Fig. 1).   All the leaves were removed except the two at the basal
node.   Then the stem was split lengthwise so that each half of the
stem contained one basal leaf and one intact bud at the apex (1, Fig. 1),
while the two buds (2. Fig. 1), in the middle node were generally
injured in the operation.   As stated, in most cases only the apical
bud grows out in such a piece of stem.   By leaving the leaf in one of

the half stems intact, while the sister leaf at the base of the other half stem is reduced in size, it is possible to find out whether the mass of the shoots regenerated at the apex of each half stem bears any relation to the mass of the two leaves. Such experiments were made by us and as a rule six or more different stems were used in one experiment.[2]

Many experiments are lost for the reason that the leaf at the base of a stem wilts or perishes much more rapidly than a leaf entirely detached from a stem; the latter may last for months while the former will usually wilt after a few weeks. For this reason it is more difficult to obtain exact results in investigating the influence of the mass of the leaf on the mass of the shoots formed at .the apex of a stem than in investigating this influence upon the mass of shoots regenerated by isolated leaves. But in watching the condition of the leaves and realizing that we can only utilize an experiment when all the leaves remain intact we are able to obtain reliable results. This source of error due to wilting and decay of the leaf attached to the base of a stem which restricts the duration of the experiments is greater when the thin summer leaves are used than when the more, fleshy and more durable winter leaves are utilized.

We shall first give the numerical results of some experiments in which only the fresh weight of the leaves and of the shoots regenerated by the stem was ascertained (Table I).

In Experiment 3 of Table I, the leaves of both half stems were left intact; 1 gm. of leaf in one set caused the production of 213 mg. of shoots in the stem, while 1 gm. of leaf of the other set produced 240 mg. of shoots in the apex of the stem. This shows the degree of accuracy to be expected in these experiments.

In the three other experiments the masses of the two sets of sister leaves varied considerably. Thus in Experiment 1 the masses of the leaves were approximately in the ratio of 1 : 6 (2.8 : 19.0); the masses of shoots produced by the two sets of stems were also approximately in the ratio of 1 : 6, namely 0.44 : 2.8. Experiment 2 gave similarly good results. The ratio of the two masses of sister leaves was approximately 1 : 5, namely 3.5 : 18.5, and the masses of shoots produced by the stem were also approximately in the ratio of 1 : 5, namely 0.7 : 3.6.

[2] Loeb, *Proc. Nat. Acad. Sc.*, 1918, iv, 117.

In Experiment 4 the two masses of sister leaves were in the ratio of almost 1 : 5, namely 2.7 : 11.9, and the masses of shoots produced by the stem were also approximately in the ratio of 1 : 5, namely 0.53 : 2.7.

Our experiments have shown without exception that the greater the mass of a basal leaf the greater the mass of shoot regenerated by the apex of the stem in equal times and under equal conditions; and wherever we were certain that the leaves remained normal during the experiment it was also possible to show that the mass of shoots produced by the apex of the stem

TABLE I·

Influence of Mass of Leaf at Base of a Piece of Stem upon Mass of Shoot Regenerated at the Apex of the Piece of Stem. Apices of Leaves Dipping in Water. Stems Suspended in Moist Air (See Fig. 2).

No. of experi- ment.	Dura- tion of experi- ment.		Fresh weight of leaves.	Fresh weight of 6 regener- ated shoots on stem.	Regenerated shoots per gm. of leaf.
	days		*gm.*	*gm.*	*mg.*
1	37	6 whole leaves......................	19.030	2.808	146
		6 sister " reduced in size..........	2.853	0.443	152
2	34	6 whole leaves	18.490	3.586	194
		6 sister " reduced in size........	3.503	0.668	186
3	26	6 whole leaves......................	7.128	1.511	213
		6 " sister leaves..................	8.142	1.963	240
4	33	6 whole leaves.......................	11.878	2.728	229
		6 sister " reduced in size........	2.740	0.530	200

varied approximately in direct proportion to the mass of the leaf attached to the base of the piece of stem.

Fig. 2 is a drawing of the appearance of a group of six pairs of half stems having a leaf at the base. The duration of the experiment was 21 days. The upper half stem with a whole leaf is always the sister piece of the half stem with a reduced leaf immediately below it. It is obvious that the stems with the reduced leaves have smaller shoots than those with whole leaves. The apices of the leaves were dipped in water, the stems were in moist air. Fig. 3 is a photographic

reproduction of the same experiment (with left and right sides reversed).

These experiments prove that *equal masses of leaves at the base of a piece of stem cause the production of approximately equal masses of shoots at the apex of the stem in equal times and under equal conditions.*
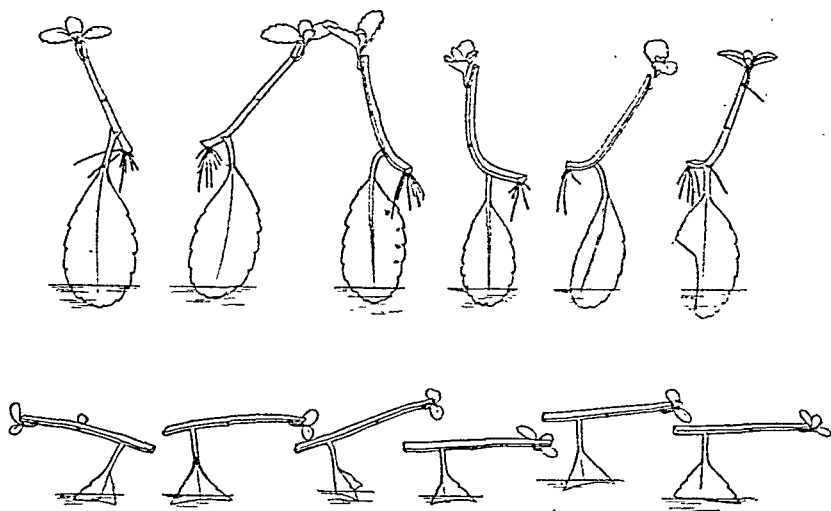
Fig. 2. Drawn after nature. The upper specimen with whole leaf and the one beneath it with reduced leaf are always the two halves of the same piece of stem split lengthwise in the way indicated in Fig. 1. The shoots at the apex of the stem with whole leaves (upper row) are considerably larger than the corresponding shoots with the sister leaf reduced in size (lower row). As a matter of fact, the masses of regenerated shoots were in proportion to the masses of the leaves attached to the base of the stems. The reader will also notice that the stems with whole leaves (upper row) have formed roots at their base, while those with reduced leaves have not yet formed any roots (though they did so later). It is also noticeable that the shoots with whole leaves show geotropic curvature, while those with reduced leaves are still straight.

Even where some of the leaves were wilted the mass of shoots produced at the apex was always greater when the mass of leaves was greater, but it was no longer possible to prove approximate proportionality though such proportionality in all probability existed.

In the experiments presented in Table II the stems as well as the leaves were suspended in moist air to eliminate the influence of ab-

Fig. 3. Photograph showing the same experiment as in Fig. 2.   (Left and right are reversed in Figs. 2 and 3.)

sorption of water by the leaf. The duration of these experiments had to be short on account of the more rapid wilting of the leaves connected with the stems.

In this case the disturbing factor of wilting is greater than when the apices of the leaves dip into water and hence the results with leaves suspended entir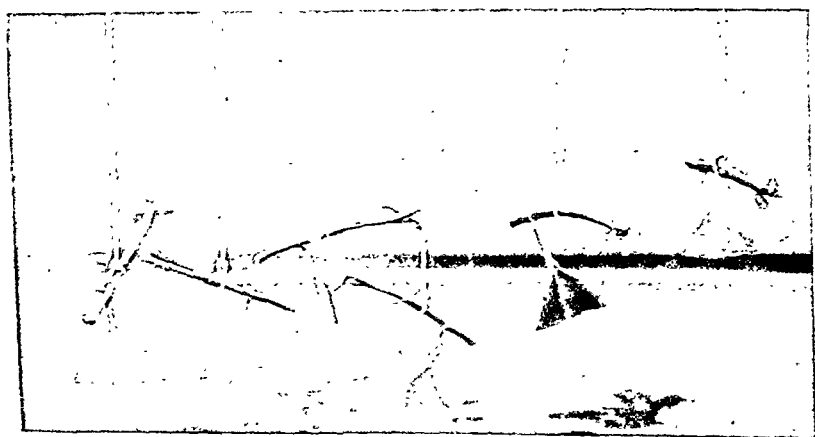ely in air show a smaller approximation to proportionality between mass of leaves and mass of shoots produced by the stem than that demonstrated in Table I, although the proportionality is not entirely obliterated.

TABLE II.

Stems and Leaves Suspended in Moist Air.

No. of experiment.	Duration of experiment.		Fresh weight of leaves.	Fresh weight of 6 regenerated shoots on stem.	Regenerated shoots per gm. of leaf.
	days		gm.	gm.	mg.
1	17	6 whole leaves........................	4.376	0.524	119
		6 " sister leaves...............	3.264	0.494	151
2	19	6 whole leaves......................	6.662	1.126	168
		6 sister " reduced in size........	2.853	0.6885	237

III.

The mass of shoot produced in an isolated piece of stem entirely deprived of leaf is small compared with that produced when a leaf is attached to the base of the stem. This was demonstrated in the following experiment.

Six stems, each containing three nodes and one leaf at the base, were split longitudinally in the way described above. One half stem contained one leaf at the base, the other had no leaf. To insure an equal water supply to the stems they were put with their cut sides on moist filter paper (Fig. 4), the leaf at the base of the one set of stems being in moist air. The duration of the experiment was 24 days.

Fresh weight of shoots produced in 6 half stems *without leaves*, 0.120 gm. (dry weight, 0.0105 gm.).

Fresh weight of shoots produced in 6 half stems *with leaves*, 2.088 gm. (dry weight, 0.2015 gm.).

Weight of 6 leaves, 8.262 gm.

The dry weight of the 6 stems without leaves was 0.286 gm. and the fresh weight was 3.935 gm. The dry weight of the 6 half stems containing a leaf was 0.688 gm. and the fresh weight 6.203 gm. We have already shown in a previous paper that the mass of an isolated piece of stem increases when a leaf is attached to it.[3]
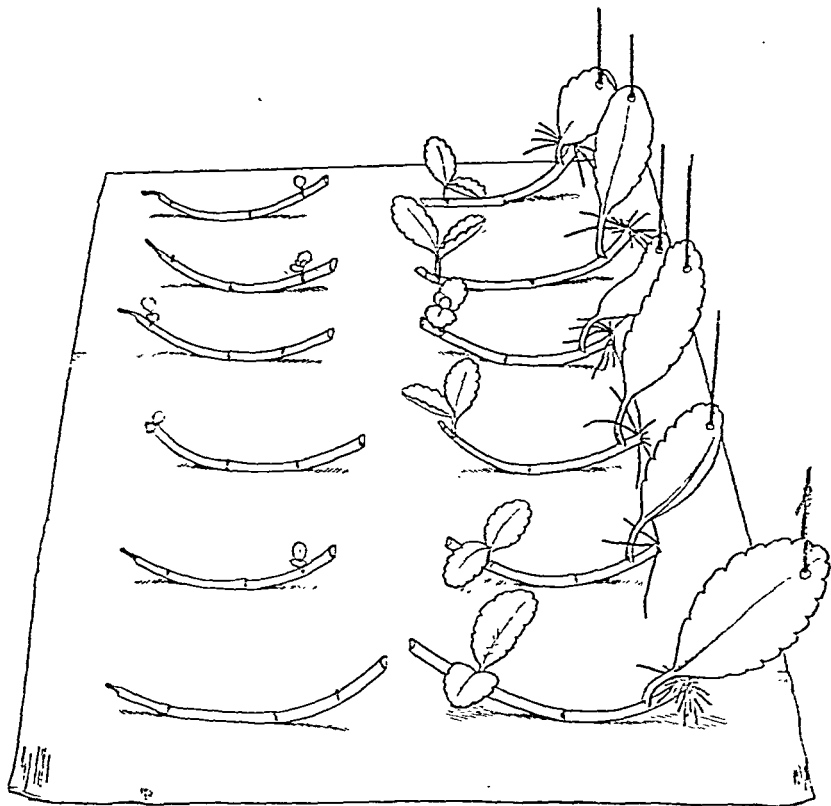
Fig. 4. Showing the enormous difference in mass of shoots regenerated by a half stem with leaf at base and the other half stem without leaf. The stems rested with their cut surface on moist filter paper.

Hence we can say that only about 5 per cent of the material of the regenerated shoots was contributed by the stem and about 95 per cent of the mass of the regenerated shoots of the stem was formed from

[3] Loeb, *Ann. Inst. Pasteur*, 1918, xxxii, 1.

material furnished by the leaf.   This is not surprising since we know that the leaf is the organ where the material for new growth in the plant is manufactured.   It is also to be expected that in pieces of stems of different mass but without leaves the mass of shoots regenerated will increase with the mass of the piece of stem.   The writer has already published experiments indicating that this is true,[4] though he has not yet made quantitative determinations to find out whether the law of proportionality holds in this case also.

IV.

The law of proportionality between mass of the leaf attached to the base of a stem and mass of shoot produced apically from the leaf can be proved for other cases also.   If we cut out a piece of stem with only one node containing two leaves such a piece possesses only two buds capable of developing into shoots; namely, one in each of the axils of the two leaves (Fig. 5).   These axillary buds grow out more rarely and more slowly than the free buds at the apex of a piece of stem. Fourteen pieces of stem with one node and two leaves each were cut out from plants and each piece of stem was split longitudinally between the two leaves.   One leaf remained always intact, the other leaf was reduced by cutting off part of the leaf (Fig. 5).   Eight of these fourteen pieces of specimens formed axillary shoots.   It seemed of interest to find out whether the mass of these shoots was approximately in proportion to the mass of the leaves.   This was the case (Fig. 5).   The duration of the experiment was 45 days.   The apices of the leaves dipped into water.

a. Weight of 8 whole leaves, 10.968 gm.   Weight of 8 shoots produced in their axil, 1.8025 gm.   Mg. of shoots produced per gm. of leaf, 164.

b. Weight of 8 reduced sister leaves, 3.586 gm.   Weight of 8 shoots produced in axil, 0.5895 gm.   Mg. of shoots produced per gm. of leaf, 164.

The mass of axillary shoots produced by each set of sister leaves was, therefore, in direct proportion to the mass of the leaves.

[4] Loeb, *Bot. Gaz.*, 1915, lx, 249.
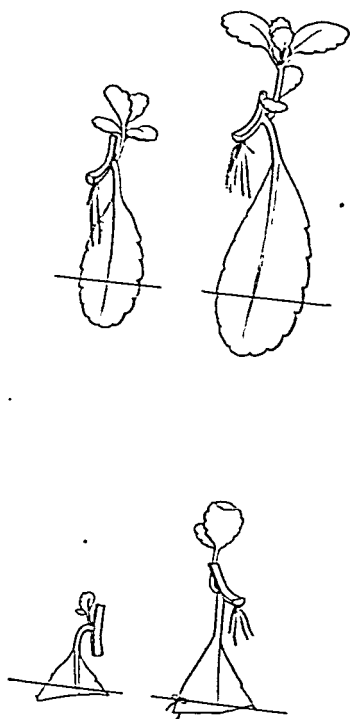
Fig. 5. Showing influence of mass of leaf on mass of shoot produced in the axil of each leaf. Each upper specimen with whole leaf and the specimen below it with reduced leaf are halves of the same stem.

V.

Since it might be argued that the leaves furnish only water for the growth of the shoots, it was necessary to find out whether the dry weight of the shoots regenerated by the stem varies also with the dry weight of the leaves attached to the base of the stem. The dry weight of the basal leaves and of the shoots regenerated by the stem was ascertained by drying these organs in an electric oven over night at 100°C. Some of the experiments mentioned in Tables I and II were used for this purpose and in Table III the experiments in which the dry weights were determined are indicated.

The leaves, therefore, do not only supply water for the regeneration of the shoots by the apex but dry matter as well. This conclusion is supported by the experiments to be discussed in the next chapter.

TABLE III.

No. of experiment.		Dry weight of leaves.	Dry weight of shoots.	Dry weight of shoots per gm. of dry weight of leaf.
		gm.	*gm.*	*mg.*
4, Table I.	6 whole leaves.......................	0.440	0.2135	0.485
	6 sister " reduced in size..........	0.1055	0.0430	0.407
2, Table II.	6 whole leaves.......................	0.3700	0.1002	0.271
	6 sister " reduced in size..........	0.1991	0.0632	0.317
1, Table II.	6 whole leaves.......................	0.3144	0.0486	0.154
	6 " sister leaves..................	0.2585	0.0450	0.177

VI.

If it is true that the leaf furnishes the material to the stem from which the regenerating shoot grows, it should be possible to show that the basal leaf in connection with a regenerating stem has (after some time) less weight than the sister leaf which is separated entirely from the stem. This can indeed be demonstrated. A piece of stem is cut from a plant and all the leaves are removed except the two leaves at the basal node of the stem. One basal leaf is entirely separated from the stem to serve as a control; the other leaf remains in connection with the stem.

Six stems, about 6 cm. long. with several nodes and with a basal pair of leaves of approximately or practically the same size were selected for the experiment. Such sister leaves of the same size have also practically the same mass as shown in previous experiments. Both the isolated leaves and the stems with one leaf attached were suspended in moist air, in the same aquarium under identical conditions of light, temperature, and moisture. After 16 days the fresh and dry weights of the two sets of leaves, namely of the detached leaves and of their sister leaves connected with the stem. were ascertained. It was found that the leaves connected with the stems weighed considerably less than the detached leaves, and the difference was far in excess of the natural variation in the weight of fresh sister leaves of equal size.

Experiment I.   Leaves and Stems in Moist Air.

	Fresh weight.	Dry weight.
	gm.	gm.
6 detached leaves, at end of experiment	8.242	0.826
6 sister leaves connected with stem, at end of experiment	6.476	0.578

Hence the leaves connected with the base of the stem had given off to the latter about one-third of their dry weight.

During this time the detached leaves formed roots and tiny shoots in some of their notches while nothing of this kind occurred in the leaves connected with the stems.   Instead twelve shoots were formed at the apices of the six stems and the twelve shoots weighed fresh 0.528 gm. and dry, 0.068 gm.   The inference is that the material for the latter came from the leaves, but apparently more material than this was given off by the leaves to the stems.   We have indeed shown in a previous paper that aside from the material for shoot formation the leaf sends material into the stem which may be used for the growth of certain tissues in the stem, resulting in callus formation, geotropic curvature, and increased thickness of the stem.[3]

The experiment was repeated with this difference, that the apices of the leaves dipped into water, while the stem and the rest of the leaves were suspended in moist air.   Both sets of leaves were in the same aquarium under equal conditions of temperature, light, and moisture, and both were of equal size and mass at the beginning of the experiment.   The experiment lasted 18 days.

Experiment II.   Apices of Leaves Dipping in Water; Stems Suspended in Moist Air.

	Fresh weight.	Dry weight.
	gm.	gm.
6 detached leaves, at end of experiment	11.159	0.791
6 sister leaves attached to base of stem, at end of experiment	4.485	0.388

Hence the leaves gave off to the stem about 50 per cent of their dry weight.

This difference is only partially accounted for by shoot production in the stem, the total shoot production amounting to 1.166 gm. fresh and 0.111 gm. dry weight.

Many more experiments than these were made and in some the leaves were weighed both at the beginning and at the end of the

experiment.[5] They leave no doubt of the fact that the basal leaf gives off· a considerable amount of its material to the stem.

We have, therefore, a right to conclude that a basal leaf gives off material to the stem, part of which is used for the formation of shoots in the stem.

Experiments were carried on in the dark and it was found that the mass of shoots regenerated under these conditions was small even when a large basal leaf was attached to the stem; and no proportionality between mass of leaf and of shoots regenerated on the stem could be found. This indicates that the products of assimilation in the leaf are part of the material used for the regeneration of the shoots on the stem.

Theoretical Remarks.

These experiments have shown that in the case of the regeneration of the leaf as well as of the stem the quantity of regeneration is determined by the mass of material sent out by the leaf and manufactured in the leaf; and possibly also to a small extent by material manufactured in the stem or circulating or stored in the stem at the time the stem was cut out from the plant.

The same simple mass law seems to hold for the quantity of roots regenerated at the basal end of the stem or basally from a leaf attached to the stem. It was very obvious in all our experiments that the mass· of roots formed in a piece of stem in a given time increases with the mass of a leaf and the roots commence to grow out later when the leaf is smaller. The writer has, however, not yet made enough quantitative measurements to permit him to state that there is a strict proportionality between mass of leaf and mass of roots regenerated.

In animals the blood and lymph play the same rôle as does the sap in plants, and we may surmise that the·quantity of sugar, aminoacids, salts, and of "accessory substances" in the body fluids determines the quantity of regeneration in animals. In an animal, regeneration may occur even when no food is taken up, and it is to be assumed that the tissues of a fasting organism constantly convert some of the material stored in the cells into sugar, amino-acids, and vitamines,

[5] We will omit these experiments here since they will be discussed in one of the following papers.

which diffuse into the blood and become available for the growth of the regenerating tissues; in other words, it is the constant hydrolysis going on in the organism which supplies the material for the growth and regeneration of fasting animals. It remains for future investigations to find out whether the ratio between mass of growth material circulating in the blood or lymph and the quantity of regeneration obeys also the simple mass law established for regeneration in *Bryophyllum*.

For those interested in the dynamics of these processes attention may be called to the fact that in our experiments the action of two masses of active material, m and m_1, in two sister leaves is compared. These masses are certain constituents of the two sister leaves, primarily the chlorophyll, and their ratio $\frac{m}{m_1}$ may be considered approximately constant throughout the duration of the experiment. Our experiments have furnished the proof that the ratio of the mass of shoot regeneration in two halves of a stem (each possessing a leaf at the base) is approximately proportional to $\frac{m}{m_1}$; or in other words, that the law controlling the quantity of shoot regeneration of the stem is a special case of the law of mass action.

SUMMARY.

1. A method is given which allows us to measure the influence of the mass of a leaf upon the quantity of shoots regenerated in an isolated piece of stem. This method consists in isolating a piece of stem with only two leaves left at the basal node and then splitting the stem lengthwise so that each half has one basal leaf. By leaving one leaf intact while the size of the sister leaf is reduced, the influence of the mass of the leaf upon the quantity of shoots regenerated by the stem can be measured.

2. This method has yielded the result that the mass of shoots regenerated at the apex of such a piece of stem increases under equal conditions and in equal time with the mass of the leaf, and is approximately proportional to the mass of the leaf.

3. Such an influence of the mass of the leaf upon the mass of shoots

growth of the regenerating shoot occurs at the expense of material furnished by the basal leaf.

4. This assumption is supported by two facts: first, that in the dark this influence of the leaf disappears more or less completely; and, second, that a leaf attached to the base of a regenerating stem after some time weighs markedly less than does a sister leaf completely detached from the stem, but otherwise under equal conditions.

5. This latter fact that a leaf when attached to the base of an excised piece of stem wilts more rapidly than when completely isolated is the reason that the proportionality between mass of a basal leaf and mass of shoot regenerated at the apex of an isolated piece of stem cannot always be demonstrated with the same degree of accuracy as the proportionality between the mass of completely isolated leaves and the mass of shoots they produce.

6. The material furnished by the leaf to the stem is not restricted to water but includes also the solutes, since not only the fresh weight but also the dry weight of the shoot regenerated by a piece of stem increases with the mass of the leaf attached to the base of the stem; and since not only the water contents but the dry weight of a leaf attached to the base of an excised piece of stem diminish when compared with the dry weight of a completely detached sister leaf.

7. The mass of shoots produced by an isolated piece of stem without leaf is small and almost negligible compared with the mass of shoots produced by the same piece of stem when a leaf of sufficient mass is attached to the base of the stem.

REVERSAL OF REACTION BY MEANS OF STRYCHNINE IN PLANARIANS AND STARFISH.

By A. R. MOORE.

(From the Physiological Laboratory of Rutgers College, New Brunswick, N. J.)

Experiments on the Planarian, Bdelloura.

It has been shown that a single excitation at a median point in the earthworm elicits a shortening of the body anterior and a lengthening posterior to that point. Active extension of the body is produced by contraction of the circular muscles, and by inhibition of contraction of the longitudinal muscles of the body. This is a case of reciprocal innervation.[1] Further, it has been found that in the earthworm, just as in vertebrates, strychnine converts inhibition into excitation. thus causing upon stimulation a contraction of the longitudinal muscles, which results in a shortening posterior to the point of stimulation.[2]

Flatworms have, in addition to longitudinal and circular systems of muscles, transverse muscle fibers. These, with the circular muscles, cause an increase in length and decrease in width and thickness of the animal upon contraction, while the shortening and thickening characteristic of the quiescent position are necessarily associated with a contraction of the longitudinal musculature and a relaxation of their antagonists. This implies also reciprocal innervation.

In order to determine whether the nervous mechanism in flatworms is similar in its reaction to that of the earthworm, the following experiments were made with specimens of *Bdelloura*. This is a marine flatworm, ectoparasitic on *Limulus*. If an active individual is touched with an instrument such as a pair of forceps, it stops locomotion, shortens and thickens; *i.e.*, the longitudinal muscles contract and the circular and transverse muscles relax. In case the animals are first

---

[1] Garrey, W. E., and Moore, A. R., *Am. J. Physiol.*, 1915, xxxix, 146.
[2] Knowlton, F. P., and Moore. A. R., *Am. J. Physiol.*, 1917, xliv, 490.

strychninized by placing them for a few minutes in a solution of strychnine sulfate (1 : 10,000), mechanical stimulation produces the opposite effect; *viz.*, extreme extension and activity of the animal.   The action of the strychnine is, therefore, to convert the inhibition of the transverse and circular muscles in the normal response into an excitation.

Experiments on the Starfish, Asterias forbesii.

Animals which orient themselves by means of contact sensitivity or stereotropism are in a state of motor equilibrium when their sensitive (ventral) regions are in contact with a surface.   When this normal relationship is disturbed, as by putting the animal on its back, rapid and exaggerated body movements take place, until by chance, the specific receptors come into contact with a surface.   Immediately from this point of contact excitatory and inhibitory impulses are sent out, the effect of which is to bring the animal without further waste effort into its normal orientation again.

In the starfish the tube feet are the stereosensitive organs.   In an inverted specimen of normal vigor all the arms show initial twisting movements.   From the arm whose tube feet first get into contact with a solid surface an excitatory impulse starts to an adjacent arm, as from A to B (Fig. 1), causing the latter to twist as so to face A ventrally and to attach its tube feet to the solid surface.   Inhibitory impulses passing from A to D and from B to C cause D and C to release any initial hold they may have had and to bring E more or less passively with them, thus turning a somersault over A and B.[3]

This reaction evidently implies reciprocal innervation.   If it were possible to reverse the functioning of this system by means of strychnine, we would have a still closer parallel with the corresponding mechanism in vertebrates.   The similarity of the reaction of the earthworm to that of the vertebrate in this respect, is perhaps to be expected since the histology of the nervous system of the annelid also shows the synaptic structures.   But because the nerve tracts of the starfish do not contain elements histologically similar, we must suppose that the strychnine acts, if it acts at all, on certain chemical elements of the neuron, rather than upon some special anatomical

[3] Moore, A. R., *Biol. Bull.*, 1910, xix, 235; *Am. J. Physiol.*, 1910, xxvii, 207.
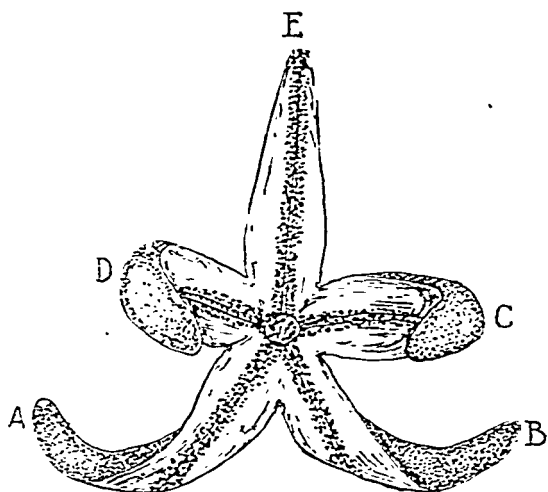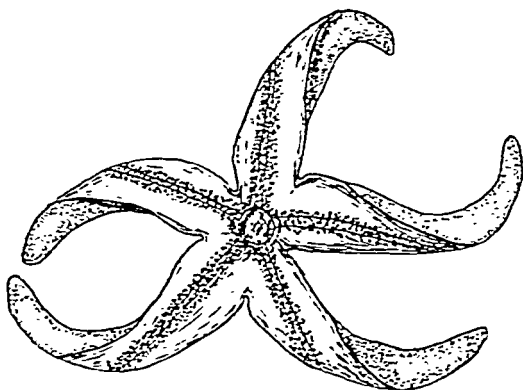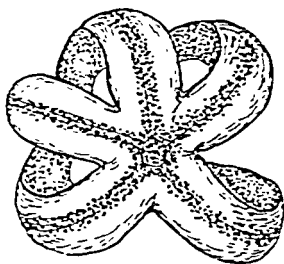
FIG. 1.

FIG. 2.

FIG. 3.

structure. It has been shown that strychnine causes hyperirritability in the starfish.[4] Proof that inhibition is reversed, *i.e.*, converted into an excitation, may be obtained by observing a strychninized starfish (strychnine sulfate 1 : 10,000) in its attempts to right itself. All of the arms take hold and retain their attachment to the bottom (Fig. 2), finally resulting in the knotted situation shown in Fig. 3. The usual inhibitory impulses which make the righting possible are no longer effective; all of the arms twist so that the tube feet maintain their hold on the bottom. Strychnine has reversed the normal inhibition.

SUMMARY AND CONCLUSIONS.

Two cases have been described, that of the marine planarian *Bdelloura* and that of the starfish *Asterias forbesii*, in which strychnine reverses reciprocal inhibition. These facts indicate that the nervous systems of these invertebrates function in a manner similar to those of the earthworm and vertebrates. Moreover, it would seem that strychnine acts upon some chemical component of the neuron which is always present in synaptic structures but which also occurs in the simpler neurons of lower forms. The fact that strychnine is without this characteristic effect on such forms as medusa and sea anemone, indicates that the nervous systems of the starfish and planarian have chemical affinities with the vertebrates which the cœlenterates do not possess.

[4] Moore, *J. Pharm. and Exp. Therap.*, 1916, ix, 167; *Proc. Nat. Acad. Sc.*, 1917, iii, 601.

LIGHT AND THE MUSCLE TONUS OF INSECTS. THE HELIOTROPIC MECHANISM.

By WALTER E. GARREY.

(From the Physiological Laboratory of Tulane University, New Orleans.)

The profound influence which light exerts upon the activities, movements, and behavior of most insects, bespeaks its deep seated effects upon the physiological processes within the neuromuscular system of these forms. Our investigations have established a direct relationship between the tonus or tension of the skeletal muscles and the illumination of the eyes of heliotropic insects, and show that the coordinated movements of these animals, like those of vertebrates, are dependent upon the maintenance of an adequate and balanced state of tonus in symmetrical groups of muscles.

It was pointed out in a preceding communication on this subject, that in the movements of heliotropic insects, the orientation was determined by the changes in muscle tension induced by unequal illumination of the two eyes.[1] These results were in conformity with the theory of heliotropism proposed by Loeb in 1888,[2] and restated by him as follows: "The mass of photochemical reaction products formed in the retina . . . . influences the central nervous system and through this the tension . . . . of the muscles. If the rate of photochemical reaction is equal in both eyes this effect on the symmetrical muscles is equal; . . . . as a consequence the animal will not be deviated from the direction in which it was moving. This happens when the axis or plane of symmetry of the animal goes through the source of light, provided only one source of light be present. If, however, the light falls sidewise upon the animal, the rate of photo-

[1] Garrey, W. E., *Proc. Nat. Acad. Sc.*, 1917, iii, 602.

[2] Loeb, J., Sitzungsber. physik.-med. Ges., Würzburg, 1888; Der Heliotropismus der Tiere und seine Uebereinstimmung mit dem Heliotropismus der Pflanzen, Würzburg, 1890.

chemical reaction will be unequal in both eyes and . . . . as a consequence the direction in which the animal moves will change."[3]

The validation of this theory rests upon presenting proof that light acting upon photosensitive organs does affect the tension (tonus) of muscles, and that asymmetrical effects are produced by unequal illumination of the eyes of heliotropic forms.   A typical example of the reflex muscle tonus produced by light is noted in the contraction of the sphincter iridis of the vetebrate eye when the retina is illuminated. The degree of contraction is proportional to the intensity of the illumination, it is quite independent of the rate of change in the illumination; furthermore the contraction is a true tonus which is maintained as long as the light affects the retina.   Exactly the same type of reaction was described by Loeb for certain sessile heliotropic animals, *Spirographis, Eudendrium*, which respond to unilateral illumination by bending toward the light.[4]   The muscles contract on the illuminated side and like the sphincter of the iris, remain in tonus as long as the light continues to act.   Loeb and Ewald and Loeb and Wasteneys[5] showed that this reaction of *Eudendrium* was proportional to the product of the intensity of the light into the duration of illumination, thus following the photochemical law of Bunsen and Roscoe.

In the case of the motile larvæ of the marine worm, *Arenicola*, which are positively heliotropic, Lillie states that there is an "increase of muscular tone under strong illumination" and that an "inequality of tone on the two sides of the body will result when one side is more strongly illuminated than the other," the muscles of the more strongly illuminated side being in stronger contraction.   Thus "heliotropic orientation is a purely muscular phenomenon."[6]   Mast[7] showed that this reaction was due to the unequal illumination of the eye spots and Garrey[1] showed that the difference in contractile state (tonus) of the muscles of the two sides of the body of *Arenicola* larvæ persists as long as the difference in the illumination of the eye spots is

[3] Loeb, The organism as a whole, New York and London, 1916, 258.

[4] Loeb, *Arch. ges. Physiol.*, 1890, xlvii, 391.

[5] Loeb, J., and Ewald, W. F., *Zentr. Physiol.*, 1914, xxvii, 1165.   Loeb, J., and Wasteneys, H., *J. Exp. Zool.*, 1917, xxii, 187.

[6] Lillie, R., *Am. J. Physiol.*, 1901, v, 59, 60.

[7] Mast, S. O., Light and the behavior of organisms, London, 1911.

maintained and is proportional to the difference in illumination of the two sides. The tonic changes produced under these conditions are entirely analogous to the changes in muscle tonus produced under the influence of a gradually increasing galvanic current and are in marked contrast to the twitch with which a muscle responds to a sudden stimulus. The analogies between electrotonus and what we may now call *phototonus*, have been considered in some detail in a previous communication.[1]

Lyon found[8] that all compensatory motions of insects were abolished by blackening their eyes, while the positions of the eyes of crustacea were so altered that he concluded that "light may cause . . . . an unequal tension of associated muscles." Ewald[9] confirmed this in a quantitative way for the orientation of the eye of *Daphnia*. Holmes[10] (1908) also describes an "increase in the tension of the leg muscles (and eye stalk of fiddler crabs) brought about by strong illumination." Delage,[11] in 1887, had already concluded that the eyes of insects seem to be their most important organs of equilibrium, and Rádl[12] believed them to be true organs of muscle tonus. Even vertebrates show that illumination of the eyes has a distinct effect upon the tonus of associated muscle groups as was demonstrated by Garrey,[13] and by Lyon's proof that the rheotropic orientation of several species of fish depended upon the optical effects of a "relative motion between the fish and its solid surroundings."[14] Loeb's[15] experiments on the compensatory motions of horned toads show a similar influence of light upon the tonus of associated muscle groups, since the effects of light and rotation, upon compensatory motions, were summed algebraically. In man, the eyes also have an effect upon the tonus of the body muscles, subordinate only to that of the internal ear and the muscle sense. This is illustrated in the so called visual nystagmus of the eyes. It may prove true that the relation of the eyes to Rom-

[8] Lyon, E. P., *Am. J. Physiol.*, 1900, iii, 86.

[9] Ewald, W. F., *Science*, 1913, xxxviii, 236.

[10] Holmes, S. J., *J. Comp. Neurol. and Psychol.*, 1908, xviii, 493.

[11] Delage, Y., *Arch. zool. exp. et gén.*, 1887, series 2, v, 1.

[12] Rádl, E., Untersuchungen über den Phototropismus der Tiere, Leipsic, 1903.

[13] Garrey, *Biol. Bull.*, 1904–05, viii, 79.

[14] Lyon, *Am. J. Physiol.*, 1905, xii, 149.

[15] Loeb, *Arch. ges. Physiol.*, 1907, cxvi, 368.

berg's sign in tabes dorsalis is due simply to a loss of muscle tonus when ocular illumination is prevented rather than to true vision; if so, it falls into the same category with the loss of tonus resulting from the degeneration of the posterior columns whereby the afferent impulses from the muscles and tendons fail.

Ewald, Lee, Lyon, Luciani and others have shown that the muscle tonus of vertebrates is maintained chiefly through afferent impulses from different parts of the otic labyrinth. Ewald states that loss of these impulses, by ablation or defect of the labyrinth, causes "an abnormal relaxation of the affected muscles, diminished energy during activity, and diminished precision of the movements in which they are concerned."[16] The insecta show no lack of muscular tone or power of precision or coordination of movements, although they possess neither otic labyrinth nor structures which suggest that they might function in any similar way. It is the intention of this report to show that the reflex effects produced by illumination of the eye of heliotropic insects establish physiological conditions in their muscles which are in every way analogous to these effects produced through the internal ear in vertebrates. The initial experiments which suggest this analogy were made by Rádl[17] but the observations of Holmes[18] (1905) had a much more direct bearing upon the problem in hand. He worked with the water scorpion, *Ranatra*, which is positively heliotropic; it seems to have been especially favorable material since both the descriptions and drawings of Holmes show striking changes of posture produced by illuminating the animal from different directions, and by blackening the eyes. Our investigations show that similar postures assumed by insects are the result of changes in the tonus of various muscle groups due to the unequal or asymmetrical illumination of the two eyes, a feature which is of general application to a great many groups of the insects.

Our experiments were conducted mainly upon the robber flies which are possessed of long legs and powerful muscles. *Proctacanthus*, (Fig. 1) was especially good material for postural studies, *Promachus*

---

[16] Ewald, J. R., Physiologische Untersuchungen über das Endorgan des Nervus octavus, Wiesbaden, 1892.

[17] Rádl,[12] p. 52.

[18] Holmes, *J. Comp. Neurol. and Psychol.*, 1905, xv, 305.

and *Deromyia* were also good, but the commoner gray forms of asilidæ did not show the reactions with the same constancy. Other forms will be mentioned when used. To obtain the desired difference in illumination, the eyes were coated wholly or in part by asphalt black varnish which forms a hard, brittle, opaque covering.

Effect of Blackening Both Eyes.—Lyon[19] noticed that house flies with both eyes blinded "show marked loss of equilibrium and often refused to fly or move in a normal manner," if "placed on their backs they would remain there for a long time without making any effort to get up." Similarly we find that *Proctacanthus* remains quiet after
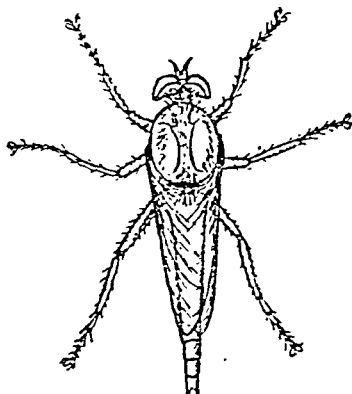
FIG. 1. Robber fly (*Proctacanthus*) under normal conditions, seen from above

blackening both eyes. When handled, the efforts to escape are feeble and unaccompanied by the vicious biting of normal robber flies. When stimulated, they move about in a most incoordinated way and usually topple over on one side or the other. Flight is without lifting power; they fall to the floor and land on their backs. Efforts to right themselves are feeble, incoordinated, and often ineffectual. When resting on a flat surface, the legs are abnormally extended and the proboscis and ventral aspect of the thorax touch the surface, as if the body weight could not be supported. Butterflies similarly settle to the surface, right themselves with difficulty, and simply flutter about, when thrown into the air, and fall to the ground.

[19] Lyon,[8] pp. 111, 112.

In the light, normal *Proctacanthus*, and flies in general, hold the body well from the surface when walking and leave only the tracks of the feet on smoked paper, but after blackening the eyes, or in the dark, some part of the body also leaves a trail. The whole experimental picture of inactivity, muscular weakness, and incoordination, when the eyes are darkened, points to a decreased neuromuscular tonus which is normally maintained reflexly by the action of light on the eyes.[20]

Effects of Blackening One Eye.—The fact that positively heliotropic flies, with one eye removed, move in circles toward the good eye was discovered by Loeb,[2] and Parker[21] found that when one eye of *Vanessa antiopa* was blackened, the animal circled in the same way both when flying and creeping and he pointed out the relation of this phenomenon to the theory of heliotropism. Holmes showed the same behavior of other forms, such as *Hyalella, Orchestia,* and *Talorchestia,* and also that the postures of the legs of the two sides of *Ranatra* were different under these conditions.[10,18] Both the circus motions and the postural changes due to unequal illumination of the two eyes, or produced by blackening one eye, are in reality shown, in some degree, by practically all heliotropic insects.

If the right eye of *Proctacanthus* is blackened, the insect circles to the left both when walking and flying. The reason for the abnormal progressive movements is evident if one examines the resting posture of the robber fly, which is more striking in this insect than in any other examined (Fig. 2). On the left side, that of the uncovered eye, the legs are all in a state of flexion, closer together than normally, and drawn well under the body, while on the other side all the legs are extended and spread out like the ribs of an open fan. The anterior leg of the normal side is adducted to the right, *i.e.* to the side of the blackened eye, and may even cross the corresponding leg of that side. The condition is a sustained, tonic one, by virtue of which the only

[20] We offer the suggestion that the restlessness of these insects in the light, and their quietness in the dark, may be a true photokinesis, that in reality both photokinesis and heliotropism have an identical physiological basis in the effects of light which reflexly increases the neuromuscular tonus and excitability, and that it is only the orienting effect of light which prevents the heliotropic forms from exhibiting photokinesis and thus coming to rest in the dark.

[21] Parker, G. H., Mark Anniversary Volume, 1903, 455.

possible movement is one in which the flexed legs pull, and the extended legs push the animal toward the illuminated eye. The legs on the side of the illuminated eye cannot be widely separated nor can those of the other side be easily approximated, thus tending to produce a wider arc of progression on the side of the blackened eye. Coupled with the asymmetrical position of the legs there is a tilting of the whole body toward the side of the unblackened eye, so that the legs on that side may be pressed to the table (Fig. 2). That the body muscles are also involved in this tilting is probable, for in some individuals, especially with strong illumination, the muscles of the
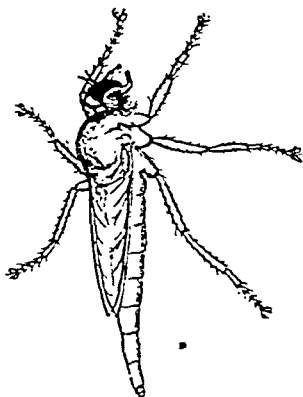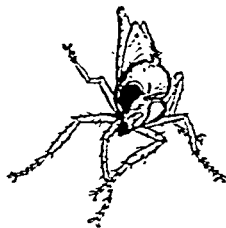
FIG. 2.   FIG. 3.

FIG. 2. Robber fly with right eye blackened, seen from above as in Fig. 1. Body tilts to the left, head rotated still farther to left. Left legs flexed, right legs extended so that, in moving, the fly circles to the left only.

FIG. 3. Robber fly with right eye blackened, viewed from the front. Posture as in Fig. 2.

abdominal segments are contracted on the side of the unblackened eye, thus producing a slight bending of the abdomen with its concavity toward that side. The head is also twisted, with the crown to the left, farther than can be accounted for by the tilting of the body (Fig. 3). This means that the head is actually rotated on the long axis of the body; at the same time the head is also turned with the blackened eye advanced, thus opening the angle with the front of the thorax on that side. Measurement of the relative tilt of the body

and twist of the head showed that when the body tilted 30° from the vertical the sagittal plane of the head inclined 50°, with a body tilt of 45° the head was inclined 75°, while in extreme cases, with very bright illumination and over reactive flies, the body frequently tilted as much as 55° or 60°, sometimes even more, and the sagittal plane of the head was horizontal.

The same postural asymmetry may be produced by simply focusing onto the left eye of a normal robber fly a beam of light from the objective of the optical system of a string galvanometer; thus the right eye is relatively dark, there is an unequal photochemical reaction in the two eyes, and asymmetry in the tonus of the muscles of the two sides of the body is produced just as it is by producing the unequal photochemical change by blackening one eye.

In general it may be stated that all butterflies, including yellows, whites, meadow-browns, coppers, skippers, and fritillaries, react in the same way. They show abnormal postures when quiet, and circle toward the unblackened eye both when walking or flying. *Circionis alope*, *Vanessa huntera*, and *Argynnis aphrodite* are good types for illustration. When resting, the wings are held together above the back and show the tilt of the body in a striking way when one eye is blackened. The average body tilt in the bright light of the laboratory was 40° as shown in Fig. 4. This figure also shows the rotation of the head by the fact that the antennæ are displaced toward the seeing eye, often both being carried to this side beyond the sagittal plane of the body; in this feature there may also be some contraction of the muscles of the antennæ, which in extreme cases carries the lower antenna parallel with, or even touching the horizontal surface on which the butterfly is resting. *Vanessa* often holds its wings spread horizontally; this is the normal position of the wings of moths; and in these cases the wing on the side of the blackened eye is elevated while the other wing is depressed and touches the surface on which the animal rests. This asymmetrical condition of tonus of the wing muscles is maintained both when resting and when walking and it is probably the cause of the circus motions when flying. The tonus of the muscles of the anterior pair of walking legs of butterflies is so altered as to produce the asymmetrical postural conditions described for *Proctacanthus*, and to an exaggerated degree. The changes in the posterior legs are not so clear.

Most of the commoner flies (*Musca, Dexia*) show reactions, when one eye is blackened, which are completely in accord with those shown by *Proctacanthus* and butterflies, and the general description already given will suffice, but the banded-eyed fly or "green-eye," *Tabanus*, is the most typical and sensitive in its responses. All the postural changes already described are well exhibited by this fly, especially the lateral flexion of the broad thin head. After blackening one of its eyes the circus motions toward the unblackened eye are made in circles of very narrow diameter, so that this fly is well adapted to the study of effects of the intensity of light, to turntable experiments, and incidentally to the study of nystactic movements of the head,
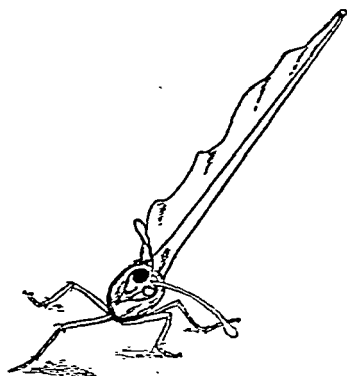
Fig. 4. Butterfly seen from the front. Shows the tilting of the body and rotation of the head toward its left after blackening the right eye.

which occur in this insect when it is rotated in both directions, even after the eye has been blackened. The nystactic movements are so easily elicited in this fly, and some others observed by the writer, that they are produced by its own spontaneous movements to either side. The drone fly, *Eristalis*, also shows marked changes of muscle tonus and forced movements due to differences in the illumination of the eyes. To produce the typical results of blackening one eye as seen in other forms, it is necessary, in this insect, also to blacken the inner half of the other eye.[22] When this is done *Eristalis* shows

[22] Several forms did not exhibit typical results after blackening one eye; for example, the vespidæ and dragon flies. *Calliphora* also is atypical in its reactions, and *Eristalis* seemed to fall into the same group. It was found on *Eristalis* that

extreme tilting of the body toward the side of the uncovered eye surface. All the legs on this side are flexed until hidden under the body and the anterior leg is adducted far toward the side on which the black has been applied and may even be visible beyond the body on that side. In preening the abdominal segments with the posterior legs, the leg on the blackened side rubs only the lateral and dorsal aspects while on the side of the functioning eye the preening is confined to the lateral and ventral aspects of the abdomen.

The fruit fly, *Drosophila*, owing to its precise heliotropic reactions would be material of choice for these studies. When one eye is brilliantly illuminated, these flies show the typical flexion of the legs on that side., The blackening of the eye is a time-consuming procedure which offers practical difficulties due to the diminutive size; one cannot be certain that injury has not been inflicted, nor that complete blackening is accurately accomplished. Nature has performed the experiment for us however. Among the types of *Drosophilæ* raised by Professor T. H. Morgan, is a type possessing one normal red eye, the other eye being white, and blind or defective. Professor Morgan called my attention to the fact that these flies move in circles toward the normal eye. He gave me the opportunity, in one instance, of convincing myself that light acting on these one-eyed forms produces the same effects as on normal flies with one eye blackened. More recently I have studied many of these flies and found that the typical asymmetry in the position of the legs is characteristic, the legs on the side of the normal eye being flexed and even concealed beneath the body, while those on the side of the defective eye were extended. The flies moved in circles from the time they were hatched until they died; they never learned to correct the abnormal physiological condition of the musculature.

after blackening one eye the typical results were obtained, in exaggerated form, by blackening the inner half of the other eye. The writer has been led to the belief that in most of these forms each eye can function like both eyes of other forms. Each eye controls the muscle tonus of symmetrical groups of muscles on *both* sides of the body. Physiologically speaking these forms are *bilaterally cyclopic*. This possibility should be borne in mind when working with forms in which it is found that blackening of one eye does not produce the expected results; thereby one of the stumbling blocks to agreement on the heliotropic mechanism will be removed.

The fact that flies which have always been blind in one eye show the same changes in the muscle tonus and the same motor reactions as other insects with one eye blackened. eliminates all idea of "avoiding reactions." The further fact that the behavior of the insects in the above experiments. and in those to be described. is due to forced motions dependent upon the conditions of muscle tonus which exist in the *resting* animals. also disposes of any relation to "trial and error."

We may summarize the results of the above experiments by stating that the tonus of the muscles depends upon the illumination of the eyes. The muscle groups affected by the right eye are different from those affected by the left eye, thus a difference in illumination of the two eyes, whether induced by illuminating one or blackening the other, produces an asymmetry when the insects are at rest. The conditions of muscle tonus thus produced are such that if the animals move, they are forced toward the side of the greater illumination. As previously pointed out,[1] the mechanism of this forced motion is the same as that in the movement of animals in which the tonus of the muscles has been produced by other means. such as passing the electric current through the body as described by Loeb and Maxwell[2] and by Loeb and Garrey.[24]

Similar tonus changes and forced motions can also be produced by ablation or defects in the internal ear of vertebrates. so that as an organ of muscle tonus, the eye of insects is comparable to the otic labyrinth of vertebrates. In elaborating this conception we shall show that there are very intimate functional analogies between the eye of the insect and the vertebrate ear, which emphasize the similarity of this relationship to muscle tonus; thus the tonus of specific groups of muscles is controlled by definite areas on the insects' eyes much as they are in vertebrates by different parts of the otic labyrinth. The following experiments will make this clear.

Effect of Blackening Part of One Eye.—This procedure has the effect, although to a lesser degree, of blackening the whole eye; more accurately, it has the effect of decreasing the illumination in that eye. The extent of the tonus changes in the muscles is, roughly speaking,

[23] Loeb, J., and Maxwell, S. S., *Arch. ges. Physiol.*, 1896, lxiii, 121.

[24] Loeb, J., and Garrey, W. E., *Arch. ges. Physiol.*, 1897, lxv, 41.

proportional to the area blackened. Thus a dot of black centrally located on one eye may produce no appreciable effect upon the position of the legs of *Proctacanthus*, although by increasing the area circumferentially until half of the eye area is blackened, postural changes are produced. But even before enough of the eye has been blackened to show the resting postural changes, circus motions toward the normal eye take place.

While it is true that this result attends blackening of an equal area on any part of the eye, there is nevertheless a marked difference in the relation of different parts of the eye to the different muscles; for example, the radius of the circus movement is less when the outer half of one eye is blackened than when the inner half is thus treated. This was put to a simple test. The outer half of the right eye was blackened and the robber fly circled to the left; the inner half of the left eye was now blackened but the fly still circled to the left, although in a much larger circle than formerly, showing only a partial neutralization of the effect. By extending the line of blackening farther out on the left eye or decreasing the area of black on the outer side of the right eye, exact neutralization could be obtained, and by continuing this process *Proctacanthus* could be made to circle to the right. The reversal of the direction of circling is thus brought about in this fly by simply altering the relative amount of light entering the two eyes although the space relation of the light field to the dark field does not change. This reaction is wholly incompatible with the idea of an "avoiding reaction."

An experiment, performed on *Eristalis*, gave results confirming the contention that in this form, each eye controls the muscles of both sides of the body. Blackening the outer half of one eye may not produce a strong tendency to circle to the opposite side. The additional blackening of the inner half of the other eye, so that corresponding visual fields of both eyes are obliterated, produces marked circus motions—much more marked than result after blackening all of one eye. Still more marked is the effect of blackening all of one eye and the inner half of the other; the circles made by *Eristalis* then have a minimal diameter, the flies often simply rotating about the vertical axis of the body if the light is intense. The explanation of this phenomenon has already been considered.

upward and backward striking the cover of a confining glass dish or looping the loop backward. Upon alighting they fall on their backs and right themselves only by turning a backward somersault. If the lower parts of the eyes are unequally blackened, there is a combination of these effects with the tendency to circle toward the eye with more surface exposed to the light.

Effect of Blackening Upper Halves of the Eyes.—The resting attitude assumed by *Proctacanthus* with the upper halves of both eyes blackened is exactly the opposite of that just described, as may be noted in Fig. 6. The body of the insect is in emprosthotonus. The abdomen is concave ventrally and there is a considerable angle between the wings and the dorsum of the abdominal segments, due to the ventral

FIG. 6. Robber fly with the upper halves of both eyes blackened. Body in emprosthotonus, head down, anterior legs flexed.

flexion of the thorax. The head too is bent down with the crown forward and the frontal aspect pressed toward the table. The anterior legs are flexed, while the posterior legs by a combination of flexion and extension of different segments, tilt the thorax upward and forward so that in some cases the robber fly literally stands on its head. Such an animal when it creeps forward strikes the head against the slightest obstacle and is precipitated in a forward somersault. In attempted flight it is not able to lift the body and crowds forward and down against the surface of the table, while if thrown into the air, it is precipitated downward in a forward somersaulting flight to the floor, where it lands on its back. In this position the fly is almost helpless, being able to right itself only if its struggles make it favorable to turn sideways; otherwise its futile efforts result in a ventral flexion of the

body in which the anterior and posterior ends of the insect meet in a ring.

Effect of Blackening Upper Half of One Eye and Lower Half of Other Eye.—By blackening the upper half of one eye and the lower half of the other eye of *Proctacanthus,* one obtains a combination of the effects described in the preceding experiments. This procedure emphasizes the fact that different parts of the eye control the tonus of entirely different groups of muscles on the two sides of the body. The resulting tonus changes produce a most bizarre type of asymmetry
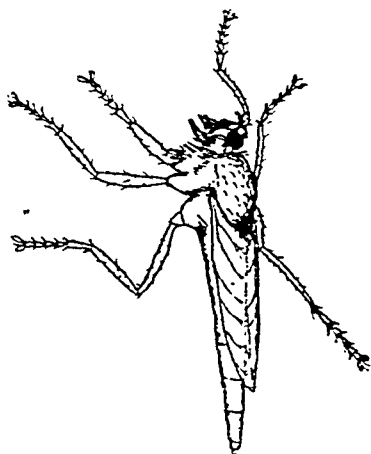
FIG. 7. Robber fly with the upper part of the left eye and the lower part of the right eye blackened. Body tilted to the right and twisted on its long axis. Head rotated to the right. Left anterior leg and right posterior leg extended. Right anterior leg and left posterior leg flexed. Moves in circles to the right.

in the position of the legs at rest, and in the character of the movements (Fig. 7). Thus with the lower half of the right eye and the upper half of the left eye blackened, there is flexion of the anterior leg of the right side and extension of its mate on the left side. There is flexion of the posterior leg on the left side and extension of the right posterior leg. The whole body is thus twisted on its long axis. The head is depressed and rotated down on the right side, that is toward the blackened area of the eye of that side, up on the left side so that the sagittal plane of head is horizontal. The tail end is twisted down on the left side.

When these insects move they do so in a most clumsy incoordinated manner, always in small circles to the right in spite of the fact that equal areas of the two eyes are blinded, indicating again the fact that different areas of the eyes control the tonus of entirely different groups of muscles. Flying movements are absolutely disorganized. The wings have no lifting power and the fly simply spins about a vertical axis, never leaving the surface of the table. If thrown into the air, the body may rotate on its long axis and gyrate in so incoordinated a way that schematic analysis is impossible. One is reminded again of asymmetrical lesions in the labyrinths of the vertebrate ears.

Effect of Symmetrical Blackening of Eyes.—Symmetrical blackening of the eyes, both outer halves or both inner halves for example, does not result in any asymmetry of posture either of the body, legs, or wings of *Proctacanthus*, provided equal areas are blackened on the two sides. There is weakening of some groups of muscles, but, the two sides being symmetrically affected, there are no forced motions either to the right or left.

Comparison of the Insect's Eye with the Otic Labyrinth of Vertebrates.—The experimental results afford irrefutable proof that the tonus of the muscles, their excitability, and force of contraction, and thus the basal conditions for coordinated movement, are dependent upon the effects of light upon the eyes of heliotropic insects. The otic labyrinth subserves these functions in vertebrates. In the latter, entirely different groups of muscles are affected by different parts of the labyrinth, and the analogy to the insect's eye is made complete by our demonstration that different parts of the eye control the tonus of different groups of muscles. Blackening the right eye affected very different groups of muscles from those affected by a similar treatment of the left eye; hence the circus motions were to the left or to the right respectively. The outer and the inner halves control different muscles. There are striking differences between the effects of blackening the upper and the lower halves of the eyes. There thus exists an *optical mosaic* in which the receptors are arranged in definite patterns which are mirrored in the groups of muscles involved in the effects of light and shade on these different areas.

It is possible even to trace, with a considerable degree of certainty, the path of the nerve impulses from the eyes to the different groups of

muscles. Such a detailed study is beside the purpose, and beyond the scope of this communication; a brief reference will suffice. Blackening one eye of positively heliotropic insects decreases the tonus and weakens the extensors of the opposite side, resulting in a flexion of the legs due to the unopposed action of the flexors. There is then at least a partial crossing of extensor nerve paths. The condition is similar to that demonstrated by operative methods on *Gelasimus pugnax* by Loeb and Maxwell.[26] The nerve paths between the eye and the flexors are not crossed; thus blackening one eye decreases the tonus and weakens the flexors on the same side and extension is unopposed. With the upper halves of the eyes blackened, the tonus of dorsal muscles is decreased and the extensors of the anterior legs are principally weakened with resulting flexion. Loss of function of the lower part of the eye weakens the flexors of the anterior legs, which become extended by the unopposed extensors, while the loss of tonus of the ventral body muscles results in opisthotonus. Other relations are easily made out.

Masked Conditions of Tonus.—The truest gauge of the physiological condition of a muscle is to be found in the character of its responses. The circus motions, which result from blackening one eye, are the result of asymmetrical conditions of the tonus of muscles of the two sides; these conditions exist in the resting muscle as is indicated by the resting postures. It often happens however that these resting postures are not in evidence, especially if the difference in illumination of the two eyes is not great, or in faint illumination after one eye has been blackened. Postures seen in one form may rarely if ever be evident in another; for example, the lateral bending of the abdomen of *Proctacanthus* often is not evident under ordinary illumination after one eye is covered. Activity however unmasks the true state of the muscle. Thus a sharp blow on the table, touching the insect with a bristle anywhere on the body, or a gentle blast of air delivered from any angle, elicits a general contraction of the body muscles without any progressive movement. In this response the expected postures are always assumed, at least momentarily; for example, a lateral bending of the abdomen of *Proctacanthus* always

[26] Loeb and Maxwell,[23] p. 134.

takes place toward the unblackened eye, and butterflies which may not lean toward the good eye, topple over toward that side if stimulated; they resist pressure against the wings from the side of the good eye but fall sideways if pushed or blown against from the side of the blackened eye. In alighting from flight such butterflies topple over toward the good eye as a result of the incoordination which results when the leg muscles are suddenly called into action. Such butterflies in righting themselves may spin about several times toward the functioning eye, although when at rest they may show no postural abnormality. These reactions demonstrate that there exists a real asymmetry in the physiological state of the muscles of the two sides, a weakness and absence of tonus which is unmasked only when a motor effort is made. We have here a perfect analogy to phenomena seen in vertebrates with certain labyrinthine abnormalities.

Asymmetrical Sensitiveness.—The maintenance of symmetrical equilibrium in the tonus and physiological state of the muscles of the two sides of the insects with which we worked, depends upon equivalent stimulation of both eyes. This is accomplished if the intensity of the illumination is the same, and the sensitiveness of the two eyes is equal. The effect of a stimulus varies directly with its intensity and also with the sensitiveness of the receptive tissue. It occurred to the writer that circus motions should result if one eye could be made more sensitive than the other; these should be in the direction of the more sensitive eye when both are equally illuminated. It was noted that *Proctacanthus* with one eye blackened shows the most pronounced postural changes and moves in circles of smallest diameter when brought from the dark room or during the early morning. The fact was established that exposure to light produces considerable fatigue of the eye and consequent weakening of the effect of light as noted in the progressive increase in the diameter of their circus movements, and that complete recovery of sensitiveness occurs after confinement in the dark. These facts suggested the expedient of simply removing the black from one eye after exposing the other to light for some hours. We were certain that the covered eye would be dark-adapted and relatively much more sensitive than the eye which had been exposed to light. Asphalt varnish hardens into a thin brittle scale and is easily cracked from the eye by a pair of fine pointed

forceps after a day or two. If the right eye had been covered, the *Proctacanthus* circled to the left, but immediately upon flaking off the covering and flooding the eye with light the direction of the circus motion was reversed and the insect circled to the right,—the side from which the black had been removed. The normal eye, owing to its previous exposure to light is fatigued; it has now become physiologically darkened although there has been no change in the illumination of that eye. The muscle tonus is affected by the greater sensitiveness of the right eye as if it were intensely illuminated. The effect may be produced by the removal of only a very small part of the black covering. The abnormal sensitiveness may persist for 2 hours or more but gradually wears off as the eye becomes light-adapted or fatigued, and the robber fly then behaves like a normal one. The experiment is a crucial one, demonstrating that the muscle tonus is proportional to the rate of photochemical reaction in the eye.

Effects of Intensity of Light upon Muscle Tonus.—Evidence has already been presented proving that muscle tonus is maintained by the action of light on the eyes and is lost in the dark, also evidence indicating that the tonus is proportional to the intensity of the illumination of the eyes. This can be demonstrated easily by the changes which result in the circus motions and postures of the insects upon varying the illumination after one eye has been blackened. A butterfly such as *Circionis alope* thus treated flew in circles with a diameter of 5 or 6 feet in the diffuse light of a room with windows on three sides, while in the sunlight the circles of flight were reduced to a diameter of 2 or 3 feet. Similarly when walking, the diameters of the circles were 2 or 3 feet when in diffuse light, but only a few inches in the sunlight. The degree to which resting butterflies leaned toward the good eye in diffuse light was distinctly increased by reflecting sunlight onto the eye from any direction. The bilateral asymmetry of the legs of *Proctacanthus* was much more marked in several individuals in the sunlight than when they were shaded, and the circles of movement had a much smaller diameter in the sunlight. *Tabanus* with one eye blackened, and *Eristalis*, with the additional blackening of the inner part of the other eye, lean far toward the seeing eye surface in sunlight and often simply circle about the tip of the posterior leg as a center, rarely in a circle with a diameter of more than 1 inch.

On the other hand, if these flies are shaded the diameter of the circles increases to 3 or 4 inches, while in the dim light of our dark room with open door, no tendency to circle was noticeable.

These effects are a function of the intensity of the illumination and are independent of all orienting effect of the light from a single source. Experiments devised to demonstrate this fact were performed by the author in 1912 and were described in lectures at Woods Hole that year.[27] The experimental insects were placed on the bottom of an illumination chamber made from a cylindrical dish, 20 inches in diameter and 12 inches deep, lined throughout with white blotting paper; the dish was covered with ground glass, and illuminated centrally from above by tungsten lights. Peep holes were provided for observation. With a 10 watt light, although one eye had been blackened, the insects moved about indifferently or at least had no difficulty leaving the floor and ascending the walls of the dish. With a 25 watt light they kept on the bottom; when a 100 watt light was used the circles were never over 6 or 8 inches in diameter, while a light of 400 watts caused a pivoting in one spot.

Results which lead to the same conclusions were obtained by partially excluding light from one eye by painting with collodion in etheralcohol solution or with shellac in alcohol. The effects of a single layer of collodion were hardly noticeable. Successive layers, especially when milky, produced progressive narrowing of the circles of movement. Shellac had the same progressive effects until the result of total blinding was produced.

The results of these experiments justify our assertion that the muscle tonus varies directly with the intensity of the illumination, and that the asymmetry of the muscular tone, upon which circus motions depend, is determined by the difference in illumination of the two eyes. The same conclusions are reached by summing the effects of gravity with light, as described below.

Experiments with Heliotropic Insects Placed upon a Vertical Surface. —Butterflies, with normal eyes, and common flies walk directly up a

[27] Similar experiments have been described by Holmes and McGraw (*J. Animal Behavior*, 1913, iii, 367) who describe "orienting effects with a constant illumination." Dr. Minnich under the direction of Professor G. H. Parker has made quantitative determinations of circus motions under similar experimental conditions; we regret that they are not yet available for reference.

vertical surface or rest with the body axis in a vertical line. When one eye has been blackened, they no longer walk vertically but veer off at an angle toward the unblackened eye. There is an algebraic summing of the tendency to follow the vertical path and to circle toward the good eye which results in the oblique path. When at rest, the body axis preserves this obliquity. Several butterflies with one eye blackened were put upon an opaque, gray, vertical screen, so placed in the laboratory that one side received the direct light from the window while the other was shaded. On the shaded side the angular deviation of the path was 15° from the vertical. When the screen was reversed, the brighter illumination from the windows caused a deviation to a path 45° from the vertical, and when placed in the sunlight, the average path in which they walked was 75° from the vertical. In the latter instance the circling tendency was often noted, and the butterflies' path might deviate to the horizontal line; they then completed a sharp turn downward toward the good eye and swung completely around again, starting the vertical ascent, but at once began to swerve off at an angle. If the butterflies are placed upon a screen, the upper half of which is in the sunlight, the lower half shaded, one is surprised at the prompt increase in the angular deviation of the path as they cross the line from the shade into the sunlight. The flies, *Tabanus* and *Eristalis*, were unable to ascend a vertical screen when it was directly illuminated by bright window light but move in circles on it; in dim light they ascend obliquely.

Experiments on a Cylinder or Spindle.—Instead of a plane, vertical surface an upright cylinder or stick may be used. The position assumed by *Proctacanthus* (Fig. 8) is oblique to the vertical and shows the typical flexion and extension of the legs due to blackening one eye. When the spindle is placed in the center of the illumination chamber described above, or when light is reflected uniformly from all sides, the effects of variations in the intensity of illumination may be determined. All heliotropic insects, with one eye blackened, ascend such cylinders obliquely (Fig. 8), and the forced motion carries them up in a uniform spiral path. Instead of measuring the angular ascent, as on the plane, vertical screen, relative effects are determined by counting the numbers of turns required to ascend a given distance. The arrangement constitutes a crude photometer, for the brighter

the light, the greater the number of spiral circuits required to make the ascent.   *Tabanus* and *Eristalis* are excellent flies for these experiments. They may be dealated or the wings glued together to prevent flying. It is convenient to place the flies inside a graduated glass cylinder to prevent escape.   Surround this cylinder with a much larger one of white mat-paper, or blotting paper, and illuminate by light reflected from above.   In one such experiment, which is quite typical, an *Eristalis* made the ascent of the inner wall of the graduate in two spirals, when illuminated by dim artificial light placed above the

FIG. 8, Position of a robber fly on a vertical spindle after blackening the right eye.   It creeps up the spindle in a spiral path to the left.

cylinder in the dark room.   In the laboratory, with the window shades down, the fly made four spiral turns.   With the shades up, twelve spiral circuits were completed in making the ascent of 12 inches. When illuminated by sunlight, the fly simply pivoted on the wall of the cylinder and was entirely unable to make the ascent.   In the latter instance the marked difference in the tonus of the muscles of the two sides, caused by the intense light, produced forced motions about the fly's dorsoventral axis.

    If an opaque spindle is arranged vertically so that one side is in the bright light, the other in the shade, insects with one blackened eye

move in very different paths on the two sides. On the shaded side the spirals are parallel and the pitch is acute, but in the bright light of the other side the fly's path is more nearly horizontal; it may become horizontal and then the fly moves in a small circle downward and may thus be trapped on the bright side of the spindle. It resumes its upward path only if, when executing the smaller circles, it is carried to the shaded side of the spindle. The different character of the paths on the two sides is only the expression of forced movements due to the different conditions of muscle tonus in light of different intensities. The facts are significant in that they explain the move-.ment of positively heliotropic insects to a light even when one eye is blackened. They do so, as the records of many observers attest, in a succession of large and small circles. In circling, the good eye is successively illuminated by, or shaded from, the luminous source. The large arcs are executed when the good eye is shaded, and the smaller arc is traversed when the good eye is coming into bright light. The effect is due only to the effects of the varying illumination upon the muscle tonus. The movements are "forced motions"—thus vanishes the mystery of "trial and error" in these instances. The mechanism of the "attraction" is the same as that in true heliotropism of flies with both eyes normal.

A study of the behavior of heliotropic insects on a turntable has yielded results confirming the conclusions drawn from the preceding experiments. These tests included observations on positively heliotropic flies, butterflies, and beetles, slow walking forms being best adapted to the end; *Tabanus* and *Eristalis* gave excellent results. Loeb[2] (1890) first described the fact that flies when rotated on a turntable, showed compensatory circus motions in a direction opposite to that of rotation. Lyon[8] showed that these disappear when the eyes are blackened, and Rádl[12] believed them to be the result of a visual fixation. These reactions, as well as the nystactic movements of the head of insects, are so suggestive of those of vertebrates, where they are due to the internal ear, that they strongly emphasize the fact that the muscle tonus of the insecta is controlled by reflexes from the eyes.

Experiments on the Turntable.—A normal fly (*Tabanus* for example) was placed in a cylinder at the center of a turntable, illuminated

equally on all sides. The ascent of the wall was made by the normal fly in a vertical line when the cylinder was stationary. When the cylinder was slowly rotated the fly circled in the opposite direction as if to maintain a vertical line of ascent; the result of the rotation however is to turn the body axis to an angle with the vertical, and the fly traverses a spiral path on the wall of the revolving cylinder. Increasing the speed of rotation (within limits) increases the number of spirals and may even cause the fly to walk horizontally or simply circle on the wall, ascent thus being rendered impossible. When this experiment is performed after blackening one eye, the fly ascends the stationary cylinder in a spiral path as described previously. Slow rotation which carries the fly toward the side of the blackened eye intensifies this forced motion, the number of spirals is increased, or the vertical component of the path is entirely nullified with a much slower rotation than in the normal fly. On the other hand, if the rotation is toward the unblackened eye, the tendency to circle in the opposite direction, which was noted in the normal fly, is still evident in the fly with the blackened eye, for it neutralizes the circus motions in part or wholly depending upon the speed of rotation. An appropriate speed may be found at which the fly no longer moves in a spiral path but ascends a vertical line on the wall of the cylinder. Faster rotation will cause ascent in a spiral in the opposite direction—a forced circus motion toward the blackened eye.

The intensity of the illumination has a decided effect on the turntable reactions of these flies. It requires a greater speed of rotation in the bright light to cause the change from a spiral to a vertical path, and conversely, if the spiral path has been changed to a vertical one by rotation toward the unblackened eye, an increase in the illumination will restore motion in a spiral path toward the good eye. This is of course due only to a reestablishment of a difference in the muscle tonus of the two sides by increase in the illumination of the unblackened eye.

Concordant results are obtained on the horizontal surface of a turntable. A fly, blinded in one eye, circles in a given radius; this radius is decreased and the fly simply turns about a vertical axis in one spot if rotated toward the blind eye. The circles are widened, or circling in the opposite direction results by rotating toward the good eye

depending upon the speed employed. The brighter the light, the easier it is to narrow the circles by rotation toward the blackened eye and the more difficult it is to neutralize the forced motion by rotation toward the normal eye. Possibly the similar behavior, on the turntable, of flies from which Loeb had removed one-half of the brain, was due to the fact that the light stimulus from one side was lacking.

In conclusion it may be stated that the condition of muscle tonus produced by blackening the eyes was not recovered from in the weeks during which many robber flies and butterflies were kept in the laboratory; as a matter of fact, the asymmetry of the muscles gradually became more pronounced and was fixed after death by the onset of rigor mortis.

SUMMARY.

The tonus of the muscles of heliotropic insects is due chiefly to the action of light; it is markedly decreased in the dark. Each eye controls the tonus of a different group of muscles on both sides of the body. Different areas of each eye likewise are related to the tonus of different muscle groups, and the relationship is entirely analogous to that of the otic labyrinth of vertebrates. Asymmetrical conditions of muscle tension are produced by any procedure which establishes an unequal photochemical reaction in the two eyes, by difference in illumination, by partial or complete blackening of one eye, or establishing unequal sensitiveness in the two eyes. The unbalanced condition of muscle tonus expresses itself in unusual postures of the resting insects, and in movements in forced paths—circus motions when one eye has been blackened. These reactions vary directly with the intensity of the illumination, as shown not only by the variation in diameters of the circles, but also by the reactions of the insects on vertical surfaces and on the turntable.

The relation of the results of these experiments to the problem of heliotropic orientation is too obvious to require detailed discussion, which could only lead to a repetition of the description of the mechanism of heliotropism which Loeb has so clearly expounded. The experiments are so completely in accordance with Loeb's muscle tension theory of heliotropism, that they are tantamount to a complete proof of it.

LUTEAR CELLS AND HEN-FEATHERING.

By ALICE M. BORING AND T. H. MORGAN.

(*From the Peking Union Medical College, and the Department of Zoology, Columbia University, New York.*)

In nearly all breeds of poultry the dorsal plumage of the male differs from that of the female in length of certain feathers, in structural features, and in most breeds in color also. In the race of Sebrights, on the other hand, the male is feathered like the female. He is said to be hen-feathered. There are other breeds, such as the Campines and Hamburghs, in which both cock-feathered and hen-feathered adult males are known.

It has recently been shown by Boring and Pearl[1] that there are groups of cells in the ovary of the hen (Fig. 1) that collect in the follicles after the egg is set free, and produce there a yellow pigment that reacts chemically in the same way as does the lutear pigment of the corpus luteum of the mammal. They call these cells lutear cells.

Boring and Pearl have also shown that the lutear cells are absent in the testes of adult male fowls.

It has been convincingly demonstrated by Goodale,[2] both for ducks and fowls, that extirpation of the ovary leads to the assumption of the full male plumage by the female. Whether the germinal material, or the connective tissue of the stroma of the ovary, or the lutear cells are responsible for the condition of the plumage of the female could not be determined by ovariotomy alone. If, however, any element should be found in the testes of the hen-feathered Sebright that was absent from other cock-feathered breeds, and like any elements peculiar to the female, then it would appear highly probable that these elements

[1] Boring, A. M., and Pearl, R., *Anat. Rec.*, 1917, xiii, 253; Pearl and Boring, *Am. J. Anat.*, 1918, xxiii, 1.

[2] Goodale, H. D., *Biol. Bull.*, 1910–11, xx, 35; *Am. Nat.*, 1913, xlvii, 159; *J. Exp. Zool.*, 1916, xx, 421.

Lutear cells   Lutear cells
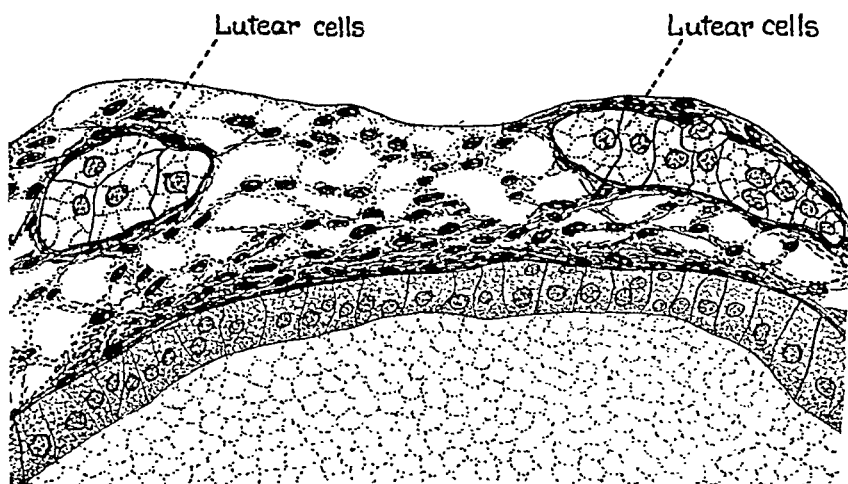
FIG. 1. Two groups of lutear cells in the theca of the follicle of the hen.

Lutear cells
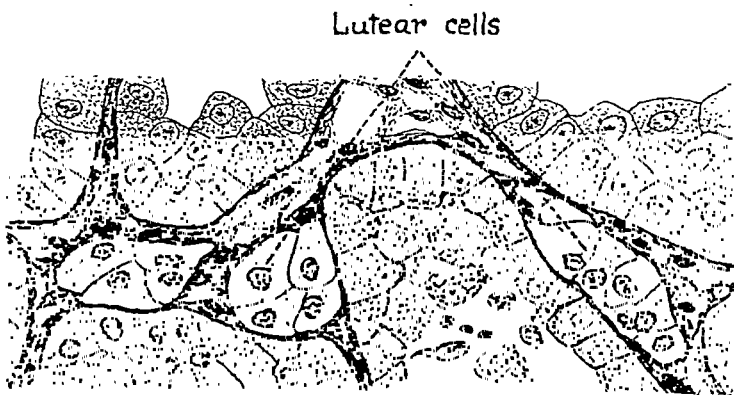
FIG. 2. Three groups of lutear cells in the connective tissue between the seminal tubules of the Sebright male.

are responsible for hen-feathering in the female as well as in the male Sebright.

A histological examination of the testis of a Sebright has shown that it contains groups of lutear cells (Fig. 2) identical in appearance with those in the ovary of the hen.   It seems practically certain that these

are the cells whose secretion suppresses in the hen and in the Sebright male the characteristic cock-feathering.

In support of this conclusion it may be pointed out that one of us has recently shown that complete removal of the testes from the male Sebright causes him to assume the plumage of the ordinary cock.[3] The result is the same as removal of the ovary from the hen (Good-

Lutear cells
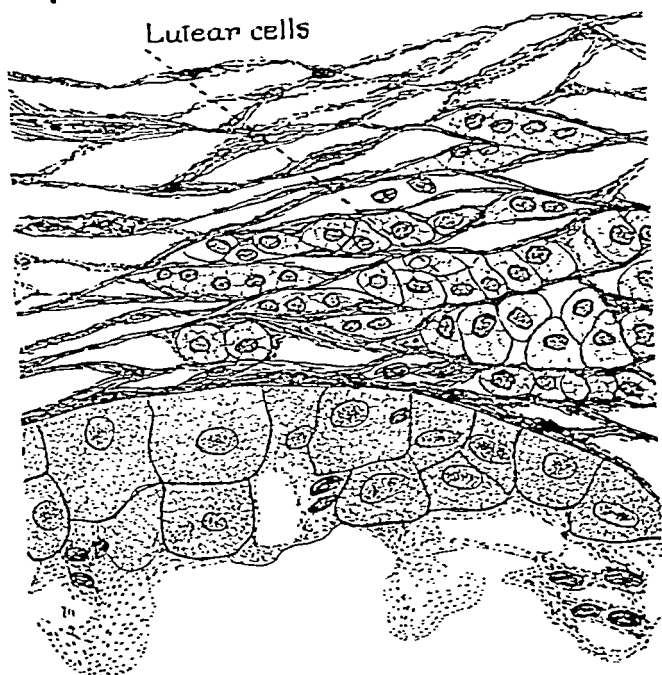
FIG. 3. Several groups of lutear cells in the regenerated testis of a Sebright male.

ale), and theoretically due in both cases to the removal of the lutear cells. The complete demonstration of the above conclusion should be obtained by implanting in an ovariotomized hen pieces of the testes of a Sebright male, producing thereby the same effect as that produced by the presence of her own ovary.

[3] Castration of the ordinary cock does not change the character of his plumage.

In Fig. 1, two groups of lutear cells, in the wall of an egg follicle of a hen, are shown.   In Fig. 2 three groups of lutear cells are seen between the tubules of the testis of a male Sebright.   In Fig. 3 lutear cells in the regenerated testis of a Sebright male are shown.   The bird had been castrated but, since it did not change the character of its plumage, after 6 months it was opened and this piece of testis was found and removed.   In Fig. 4 a piece of the testis of a hermaphrodite bird described by Boring and Pearl shows a large group of lutear cells.
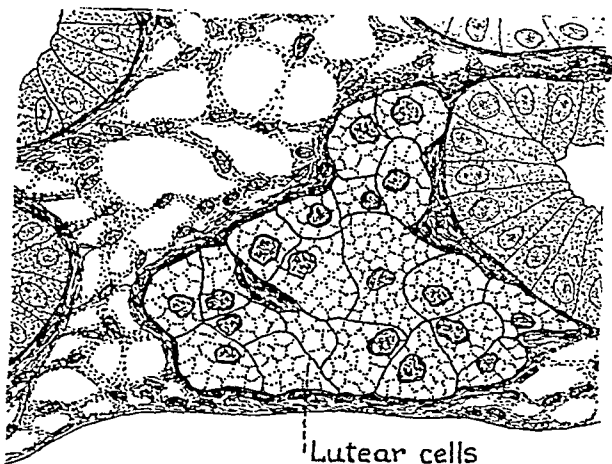
Lutear cells

FIG. 4. A group of lutear cells in a hermaphrodite fowl (from Boring and Pearl).

CONCLUSIONS.

The experimental evidence had made clear that some substance is produced in the testis of the male Sebright that suppresses in him the development of the secondary sexual plumage of the cock of his species.   The detection in his testis of lutear cells like those in hens makes the conclusion highly probable that it is these cells that cause the suppression of cock-feathering in both the Sebright male and in hens of all fowls.   Genetic work by Morgan[4], had shown that one or two Mendelian factor-differences are responsible for hen-feathering

[4] Morgan, T. H., *Am. Nat.*, 1917, li, 513; *Proc. Soc. Exp. Biol. and Med.*, 1915–16, xiii, 31.

in the Sebright. These factor-differences produce their effects through the testes. The presence of these genetic factors, we now see, causes the testes of the Sebright to produce a kind of secretory cell that is ordinarily only produced in the female, or possibly to a slight extent in young males (Boring), or in numbers insufficient to suppress the male plumage in the testes of some ordinary cock birds (Reeves[5]).

[5]Reeves, T. B., *Anat. Rec.*, 1915, ix, 383.

MONOGRAPHS OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH.

Under the head of *Monographs of The Rockefeller Institute for Medical Research* are published from time to time scientific papers which are so extensive, or require such elaborate illustrations, as to render them unsuitable for current periodical issues. *Monographs* will be sent post paid on application, at $1.00 each, payable in advance by check or money order.

Monograph No. 1. (Issued June 30, 1910.)

SIMON FLEXNER and J. W. JOBLING. Studies upon a transplantable rat tumor. Plates 1–16 J. W. JOBLING. The biology of a mixed tumor of the rat. MAUD L. MENTEN. Experiments on the influence of radium bromide on a carcinomatous tumor of the rat. J. W. JOBLING. Spontaneous tumors of the mouse. Plates 17–28. J. W. JOBLING. Transplantation experiments in *Macacus rhesus* with a carcinomatous teratoma from man.

Monograph No. 2. (Issued February 15, 1911.)

WILLIAM TRAVIS HOWARD and OSCAR T. SCHULTZ. Studies in the biology of tumor cells. Plates 1–6.

Monograph No. 3. (Issued March 15, 1911.)

BENJAMIN T. TERRY. Chemo-therapeutic trypanosome studies with special reference to the immunity following cure.

Monograph No. 4. (Issued June 24, 1912.)

FRANCIS W. PEABODY, GEORGE DRAPER, and A. R. DOCHEZ. A clinical study of acute poliomyelitis. Plates 1–13.

Monograph No. 5. (Issued September 27, 1915.)

J. P. SIMONDS. Studies in *Bacillus welchii*, with special reference to classification and to its relation to diarrhea.

Monograph No. 6. (Issued January 31, 1916.)

JAMES L. STODDARD and ELLIOTT C. CUTLER. Torula infection in man. Plates 1–9.

Monograph No. 7. (Issued October 16, 1917.)

OSWALD T. AVERY, H. T. CHICKERING, RUFUS COLE, and A. R. DOCHEZ. Acute lobar pneumonia. Prevention and serum treatment. Plates 1–3.

Monograph No. 8. (Issued July 31, 1918.)

ERNEST C. DICKSON. Botulism. A clinical and experimental study. Plates 1–10.

Monograph No. 9. (In press.)

JAMES HOWARD BROWN. The use of blood agar for the study of streptococci.

THE JOURNAL

OF

GENERAL PHYSIOLOGY

EDITORS

JACQUES LOEB   W. J. V. OSTERHOUT

VOLUME I, NO. 2   NOVEMBER, 1918

PUBLICATIONS OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

THE JOURNAL OF EXPERIMENTAL MEDICINE

EDITED BY

SIMON FLEXNER, M.D.

The Journal of Experimental Medicine is designed to cover the field of experimental medicine. It is issued monthly, two volumes of over 600 pages each appearing in a year. The subscription price per year is $5.00 (two volumes); single copies, 75 cents.

THE JOURNAL OF BIOLOGICAL CHEMISTRY

EDITED BY

H. D. DAKIN, New York, N. Y.   A. N. RICHARDS, Philadelphia, Pa.
E. K. DUNHAM, New York, N. Y.   DONALD D. VAN SLYKE, New York,
LAFAYETTE B. MENDEL,   N. Y.
    New Haven, Conn.   CLARENCE J. WEST, New York, N. Y.

The Journal of Biological Chemistry is issued monthly, and since its foundation in 1905 thirty-five volumes of approximately 550 pages each have been completed. The subscription price is $3.00 per volume to subscribers in the United States and Canada; $3.25 to those in other countries. The number of volumes issued yearly averages four.

THE JOURNAL OF GENERAL PHYSIOLOGY

EDITED BY

JACQUES LOEB, New York, N. Y.   W. J. V. OSTERHOUT, Cambridge,
    Mass.

The Journal of General Physiology is devoted to the explanation of life phenomena on the basis of the physical and chemical constitution of living matter. Information as to contributions and subscriptions is given in full on the back cover.

STUDIES FROM THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

The published results of investigations conducted in the Institute, or under its grants, are republished at irregular intervals in volumes designated *Studies from The Rockefeller Institute for Medical Research*. Price, $2.00 per volume, payable in advance. A special rate of $1.00 per volume is made to those who subscribe to *The Journal of Experimental Medicine, The Journal of Biological Chemistry*, or *The Journal of General Physiology*.

Owing to the difficulties placed in the way of the printing trades by war conditions, *The Journal of General Physiology* desires to cooperate with the printer by eliminating unnecessary labor in the setting up of manuscripts. To accomplish this object, parts of the papers to be printed in different styles of type should be separated in the manuscript. For example, every experiment, table, or quotation of over five lines should begin on a new sheet. When the text is resumed, a fresh sheet should be started. Separate sheets should be used for the running headlines, title, author, foot-notes, and bibliography. The name of the laboratory where the work was done together with the words, "(Received for publication, , 1918)," should also be written on a separate sheet.

The entire manuscript should be copied with triple spacing.

In general the forms for headings, tables, references, etc., outlined in the "Suggestions for the preparation of manuscripts," issued by the Publication Department, should be followed. A copy of the "Suggestions" will be found on the back pages of the 25-Volume Index of *The Journal of Biological Chemistry*. We are glad to mail additional copies to contributors.

The *Journal* requests that, when possible, authors prepare their manuscripts in conformity with these suggestions.

STUDIES ON BIOLUMINESCENCE.

VII. REVERSIBILITY OF THE PHOTOGENIC REACTION IN CYPRIDINA.

BY E. NEWTON HARVEY.

(*From the Department of Marine Biology, Carnegie Institution of Washington, Washington, and the Physiological Laboratory, Princeton University, Princeton.*)

CORRECTION.

On page 23, Vol. I. No. 1. September, 1918, line 1 of the title of the article, for *thyroid* read *thymus;* the same correction should be made in the Contents.

photophelein. Whenever two such non-luminous solutions are mixed light appears.

On the grounds of method of preparation, relation to temperature, and dialysis, I regarded photogenin as comparable to luciferase and photophelein as comparable to luciferin, two photogenic substances described by Dubois[2] in the beetle, *Pyrophorus noctilucans*, and in the mollusc, *Pholas dactylus*. Dubois believes that luciferase is an oxidizing enzyme which oxidizes luciferin, an oxidizable substance, with light production. Neither luciferase nor luciferin alone in solution can produce light but light appears if solutions of the two are mixed and continues as long as any luciferin remains unoxidized. Dubois has also been able to produce light by oxidizing luciferin (alone) with a small crystal of $KMnO_4$, by H_2O_2 (with or without blood containing

---

[1] Harvey, E. N., *Am. J. Physiol.*, 1917, xlii, 318.
[2] Dubois, R., *Compt. rend. Soc. biol.*, 1885, xxxvii, 559.

hemoglobin), BaO_2, PbO_2, and other oxidizing agents. Through the kindness of Professor Dubois I have received some material of *Pholas dactylus* preserved in sugar and I can confirm his results on the effect of $KMnO_4$ and other oxidizing agents in producing light with luciferin of *Pholas*. I have likewise repeated my own experiments with the photophelein of *Cypridina* using a whole series of oxidizing agents applied in the same way, as with the luciferin of *Pholas* and, as previously, have failed to obtain any light with this substance.[3] The difference in our results is, therefore, not to be referred to a difference in method of experiment but to a difference in the substances themselves.

I found also that if one takes a concentrated solution of photogenin filtered through a porcelain or silicious filter candle to remove all granules and cell fragments and adds to it a little saponin powder, or amyl alcohol or NaCl or other inorganic salt crystals or tissue extracts of certain invertebrate non-luminous animals, that light would appear. Because NaCl could not possibly be oxidized by photogenin (= luciferase)—or any other substance—and because of my inability to make photophelein (= luciferin) luminesce with oxidizing agents, I regarded the photogenin itself as the source of the light and the oxidizable body. I have compared photogenin to zymase and photophelein to the coenzyme of zymase, believing that we are dealing with a system similar to that of the enzyme-coenzyme system of yeast. Hence the name photophelein or body assisting in the production of light.

[3] The following oxidizing agents (added ,where possible, in minute crystal or powder form) all gave light with *Pholas* luciferin, but no light with *Cypridina* luciferin: $KMnO_4$, $K_2Cr_2O_7$, PbO_2, Na_2O_2, BaO_2, MnO_2, $K_3Fe(CN)_6$, $K_2S_2O_8$, $Na_2B_4O_8$, and H_2O_2. The following oxidizing agents gave no light with either *Pholas* luciferin or *Cypridina* luciferin: K_2CrO_4, CrO_3, $KClO_3$, $KClO_4$, $FeCl_3$, KNO_3, Cl or Br water, I in KI, Na hypochlorite, hypobromite, or hypoiodite, colloidal Ag or Pt, benzoyl peroxide, potato or turnip juice, or blood containing hemoglobin or hemocyanin. If H_2O_2 in addition to the oxidizing agent is added to *Cypridina* luciferin, no light appears except a faint momentary flash with Na hypochlorite and hypobromite. As this faint flash also appears with thoroughly boiled extracts of *Cypridina*, lacking luciferin, it can have no significance. If H_2O_2 in addition to the oxidizing agent is added to *Pholas* luciferin the light is in some cases brighter than with H_2O_2 alone.

I now believe that under the term photophelein I have previously included two separate substances. One of these is the thermostabile dialyzing substance extracted from *Cypridina* by hot water. Although this substance cannot be oxidized with light production by oxidizing agents, it does oxidize spontaneously (also without light production) in the air and loses its power of producing light with photogenin. In the absence of air its solutions are stabile for months. Once oxidized it can again be reduced and will again give light if photogenin is added. It is therefore an oxidizable material and, I believe, similar to the luciferin of *Pholas*. I propose therefore to use Dubois' word luciferin for the thermostabile dialyzing substance of *Cypridina* in place of photophelein and luciferase for the thermolabile non-dialyzing substance in place of photogenin. The source of the photogenic substances can be designated by prefixing the name of the animal as *Cypridina* luciferin, *Pholas* luciferin, etc. I suggest also that luciferin when oxidized be designated oxyluciferin.

Luciferin is found only in luminous animals. In non-luminous animals and probably also in luminous animals there is a second substance which I have formerly included in the term photophelein, and which may be properly so called, that acts in a manner similar to saponin, NaCl crystals, etc., upon the extract of *Cypridina* which has stood until the light disappears. When we allow a *Cypridina* extract containing luciferin and luciferase to stand, the luciferin is not completely oxidized, even though the extract is thoroughly aerated, but some of it is bound (adsorbed or combined?) by other substances in the extract. The saponin, NaCl crystals, and extracts of non-luminous animals act by setting free the bound luciferin which is then oxidized and light appears. I suggest that the term photophelein be applied to these substances in tissue extracts. They are not destroyed by boiling. On standing some are stable while others are unstable.

The best way to rid a luciferase solution of the bound luciferin is to shake it thoroughly with chloroform. Such a solution will give no light with extracts of non-luminous animals or saponin, NaCl crystals, etc., but a brilliant light with *Cypridina* luciferin.

An insight into the *modus operandi* of saponin, NaCl crystals, or photophelein may be gained from the following experiments. Both luciferin and luciferase are adsorbed by many finely divided precipi-

tates and colloidal particles, such as bone-black, $Fe(OH)_3$, kaolin, and others. If we take a colloidal $Fe(OH)_3$ solution of the proper concentration (which can only be determined by experiment), add some dilute luciferase to it, and then after a minute luciferin, no light will appear. This is because the luciferase has been completely adsorbed by the colloidal $Fe(OH)_3$, for if we now add some dilute luciferase to the above mixture light will appear but it will very quickly disappear because the new luciferase added is again very rapidly adsorbed, but not so rapidly adsorbed that we fail to get light at first. On adding more luciferase we may again get a momentary light but the additions cannot be made indefinitely because we finally reach a point where the colloidal $Fe(OH)_3$ has become saturated with luciferase and then the mixture glows for a considerable time. It is obvious that for this experiment to succeed there must be more luciferin present than can be completely adsorbed by $Fe(OH)_3$ and so little luciferase present that it is completely adsorbed by the $Fe(OH)_3$. Suppose we have a mixture of $Fe(OH)_3$, luciferase, and luciferin complying with the above conditions. Can we in any way remove the luciferase from its adsorbed condition on the colloidal $Fe(OH)_3$? This might theoretically be done in two ways and we actually find in practice that both methods are possible. Anything which precipitates the colloidal $Fe(OH)_3$ will decrease the surface available for adsorption of luciferase and if the surface area is sufficiently decreased some luciferase may be forced into solution again where it is able to oxidize the luciferin. If we add NaCl crystals to the colloidal $Fe(OH)_3$ + dilute luciferase + more concentrated luciferin mixture, the $Fe(OH)_3$ is precipitated and light appears. If in place of NaCl crystals we add a trace of saponin, the colloidal $Fe(OH)_3$ is not precipitated but light also appears. This is an example of the second method of removing luciferase from an adsorbed condition; namely, by using a material (saponin) which is more strongly adsorbed than the luciferase and which is able to replace it as adsorbed body. I believe these to be the explanations of the effect of NaCl crystals, saponin, etc., in giving light with luciferase solutions, except that the luciferase is in excess and a small amount of adsorbed (or combined) luciferin is present which is liberated by NaCl or saponin and gives light with luciferase. Photophelein probably acts in a manner analogous to the saponin.

I have considered the thermostabile, dialyzing substance as similar to the luciferin of. *Pholas* despite the fact that Dubois reports *Pholas* luciferin destroyed at 70°C., whereas *Cypridina* luciferin is only destroyed by several minutes boiling in an open beaker. I find that this destruction of *Cypridina* luciferin on short boiling is due to the increased rate of oxidation at the boiling point and that no destruction of *Cypridina* luciferin will occur if boiled in an atmosphere of hydrogen.[4] *Cypridina* luciferin is truly thermostabile but is oxidized to oxyluciferin on boiling in the air. We may say that *Pholas* luciferin is *similar* but certainly not *identical* with *Cypridina* luciferin. If so, we should expect to obtain light on mixing *Pholas* luciferin and *Cypridina* luciferase, yet no light appears. Neither is there light on mixing *Cypridina* luciferin and *Pholas* luciferase, although the *Pholas* luciferase prepared from the material which Dubois sent me gave a rather faint light with *Pholas* luciferin.[5]

We have, therefore, at least three substances concerned in light production: luciferin, luciferase, and photophelein. Luciferin is a body oxidizing with light production, dialyzable, and relatively resistant to heat. Luciferase is destroyed by boiling, non-dialyzable, and accelerates the oxidation of luciferin. While it may be used up in the reaction if mixed with a sufficient quantity of luciferin, luciferase has

[4] I have endeavored to repeat this experiment with the luciferin of *Pholas* sent me by Professor Dubois, but without success. *Pholas* luciferin boiled in a current of hydrogen for 15 minutes would give no light when a crystal of KMnO$_4$ was added. The hydrogen was produced in a Kipp generator and may have contained a little air. In my experience short (20 to 40 seconds) boiling of *Pholas* luciferin does not completely destroy its power of producing light when a crystal of KMnO$_4$ is added.

[5] I believe the faint light obtained on mixing *Cypridina* luciferin and fire-fly or *Noctiluca* luciferase and *vice versa*, recorded in my former paper (*Am. J. Physiol.*, 1917, xlii, 328) where luciferin is called photophelein and luciferase is called photogenin, is not due to the oxidation of luciferin by luciferase of the second species but is due to the presence of photophelein. I am led to this conclusion because the light is so faint, but cannot be sure until the cases are reinvestigated. The mixing of luciferin and luciferase of different species or genera of luminous ostracods, especially if the colors of their luminescence differed, would shed considerable light on this interesting question of specificity. A non-luminous Japanese species of *Cypridina* does not contain either luciferin or luciferase but it does contain photophelein.

many of the characteristics of an enzyme and certainly as much right to be called an enzyme as the peroxidases of plants which are also used up in the oxidation process.   The *Cypridina* luciferase reaction appears to be specific to an extraordinary degree.   Of many tried I have found no substances or plant or animal extracts which can take the place of luciferase[6] nor any substances[7] or plant or animal extracts[8] which can be oxidized with light production by luciferase.   The light recorded with various extracts of luminous and non-luminous animals in my former paper is to be referred to the presence of photophelein, the third substance concerned in light production which probably acts by assisting the luciferin-luciferase reaction in the manner already suggested.   Let us now turn to the oxidation product or products of luciferin.

When luciferin is oxidized it must be converted into some substance or substances and I believe this change involves no fundamental destruction of the luciferin molecule, as it is a reversible process.   I

[6] I have tried the blood or extracts of many species of animals or plants including those containing strong oxidizing enzymes both with and without H_2O_2 and have always failed to obtain light with *Cypridina* luciferin.   Among others the juice of Indian pipe (*Monotropa*), potatoes, and turnips (containing strong oxidases and peroxidases), the blood of the ox and a worm (*Arenicola*) (containing hemoglobin), the blood of the squid (*Loligo*), *Limulus* and *Sycotopus* (containing hemocyanin), and extracts of *Chætopterus* (a luminous annelid), and the mollusc, *Unio*, (rich in manganese) were tried.   Dubois reports that he has obtained light on mixing *Pholas* luciferin with the blood of divers molluscs and marine crustaceans (*Ann. Soc. Linn. Lyons*, 1913, xl).   I can confirm this statement for an extract of *Unio*, but obtained no light with *Limulus* blood, *Sycotopus* blood, squid (*Loligo*) blood, or turnip or potato juice and *Pholas* luciferin.   Evidently *Pholas* luciferin is much more readily oxidized with light production than *Cypridina* luciferin.

[7] The following oxidizable substances have been tested: æsculin, lophin, bergamot oil, pyrogallol, gallic acid, aniline, adrenalin, phenol, α-napthol, *p*-phenylenediamine, ortol, orcinol, hydrochinone, resorcinol, pyrocatechol, tannin, benzidine, gum guaiac, amidol, α-napthylamine, and the chromogen of the false indigo plant (*Baptisia*).   Luciferase, with or without H_2O_2, will not accelerate the oxidative color change in any of the above compounds.

[8] I have regularly obtained a fair light on mixing luciferase well shaken with chloroform to set free any bound luciferin and boiled potato or turnip juice or boiled *Limulus* blood.   The light is especially marked about the coagulum in the boiled *Limulus* blood.   The significance of these results is not apparent.

shall speak of the principal if not the only product formed as oxy-luciferin. Most observers have considered a rather fundamental change to occur when the photogenic substance is oxidized. Thus the crystals of xanthine or some related substance in the reflecting layer of the fire-fly have been regarded as the oxidation products of the luminous material thought to be nucleoprotein. Dubois[9] regards luciferin as a protein and states that it forms the same oxidation products as other proteins, amino-acids being mentioned as possible substances formed. It should be pointed out in this connection that the formation of amino-acids from proteins involves no oxidation but an hydrolysis.

If we assume that the oxidation of luciferin changes the molecule but slightly, we at once think of comparing the change luciferin $\rightleftarrows$ oxyluciferin with the change reduced hemoglobin $\rightleftarrows$ oxyhemoglobin. The condition is, however, not so simple as this, for oxyhemoglobin will again give up its oxygen providing the partial pressure of oxygen is sufficiently low, whereas oxyluciferin will not do this. We cannot reduce oxyluciferin solution by exhausting the oxygen with an air pump.

There is another oxidation reduction system which can also be easily reversed, but not by merely removing the oxygen—that is the reduction of a dye such as methylene blue to its leuco base. I believe the change which occurs when luciferin is oxidized is similar to that which occurs when the leuco base of methylene blue or sodium indigosulfonate is oxidized to the blue dye.

My attempts to reduce the oxidation product of luciferin started from the observation that if one places a clear solution of luciferase in a tall test-tube, although it may give off no light at first when shaken, after standing a day or so a very bright light would appear on shaking. This was especially true when the luciferase had become turbid and ill smelling from the growth of bacteria. Thinking that the bacteria produced a substance which could be oxidized by the luciferase, I tried growing bacteria and also yeast on appropriate culture media and after some days of growth mixing the culture media containing the products of bacterial or yeast growth with luciferase,

[9] Dubois, *Ann. Soc. Linn. Lyons*, 1914, lxi, 169.

expecting to obtain light. But no light appeared. However, if a little crude luciferase solution was added to the bacterial or yeast cultures and then allowed to stand for some hours, light appeared whenever they were shaken. Indeed such cultures behaved much as a suspension of luminous bacteria which has used up all the oxygen in the culture fluid and will only luminesce when, by shaking, more oxygen dissolves in the culture medium. Realizing that in bacterial cultures in test-tubes anaerobic conditions soon appear, and also the strong reducing action of bacteria upon many substances (for instance nitrates or methylene blue) under anaerobic conditions, it occurred to me that the bacteria might be utilizing the oxygen of the oxidation product of luciferin, reducing it to luciferin again. We must remember that since crude luciferase solution is a cold water extract of a luminous animal allowed to stand until all the luciferin has been oxidized, it must contain oxyluciferin as well as luciferase and will give light if the oxyluciferin is again reduced and oxygen admitted. This appears to be the correct explanation of the above experiments.

Not only bacteria but also tissue extracts have a strong reducing action in absence of oxygen. Thus, muscle tissue stained in methylene blue will very quickly decolorize (reduce) the methylene blue if oxygen (air) is kept away, but the blue color immediately returns if air is admitted. Oxyluciferin, *i.e.* a solution of luciferin which has been completely oxidized by boiling or standing in air until it no longer gives light with luciferase, if mixed with a suspension of ground frog muscle and kept in a well filled and stoppered test-tube for some hours, is reduced to luciferin and gives a bright light if now poured into luciferase solution. Frog muscle suspension alone or oxyluciferin alone gives no light with luciferase, nor will a mixture of frog muscle suspension and oxyluciferin, if shaken with air for several hours. Only if this last mixture is kept under anaerobic conditions is the oxyluciferin reduced.

The reducing action of tissues is said to be due to a reducing enzyme (reducase or reductase), itself composed of a perhydridase and some easily oxidized body such as an aldehyde.[10] In the presence of the

[10] Bach, A., *Biochem. Z.*, 1911, xxxi, 443; xxxiii, 282; 1912, xxxviii, 154; 1913, lii, 412.

perhydridase the oxygen of water oxidizes the aldehyde and the hydrogen set free reduces any easily reducible substance which may be present. There is a perhydridase in fresh milk, spoken of as Schardinger's enzyme,[11] which is destroyed by boiling. If some aldehyde is added, fresh milk will reduce methylene blue to its leuco base or nitrates to nitrites, upon standing a short time. If shaken with air, the blue color returns. There is no reduction unless an aldehyde is added or unless some boiled extract of a tissue such as liver is added. The boiled liver extract has no reducing action of its own but supplies a substance similar to the aldehyde which has been spoken of as a coenzyme. Milk will reduce methylene blue without aldehyde if bacteria are present in large numbers. There is also no reduction if the milk, methylene blue, and aldehyde are agitated with air. The temperature optimum is rather high, 60–70°C.

I find that milk is a favorable and convenient medium for the reduction of oxyluciferin and that it acts without the addition of an aldehyde or the presence of bacteria. There is probably a substance acting as the aldehyde in the luciferase-oxyluciferin solution. No light appears if milk is added to a luciferase-oxyluciferin solution, but if the mixture is allowed to stand in absence of oxygen, light will appear when air is admitted. The air can be conveniently kept out by filling small test-tubes completely with the solution and closing them with rubber stoppers.

Oxyluciferin may also be readily reduced by the use of the blood of the horseshoe crab (*Limulus*) allowed to stand until bacteria develop.[12] This experiment is of special interest because the blood contains hemocyanin which is colorless in the reduced condition and blue in the oxy-condition. The color change thus serves as an indicator of the oxygen concentration in the blood. A sample of foul smelling *Limulus* blood full of bacteria will become colorless on standing in a test-tube for 10 or 15 minutes but the blue color quickly returns if shaken with air. Such a blood has the power of reducing oxyluciferin through the activity of the bacteria which it contains. Fresh blood has very little if any reducing action.

[11] Schardinger, F., *Chem. Ztg.*, 1904, xxviii, 704.
[12] Alsberg, C. L., *J. Biol. Chem.*, 1915, xxiii, 495.

As almost all animal tissues contain reductases, it is not surprising to find that a freshly prepared and filtered extract of *Cypridina* containing oxyluciferin and luciferase which gives no light on shaking, will, on standing in a stoppered tube for 24 hours at room temperature, give light when air is admitted.[13] While this may be due to the development of bacteria with a reducing action it does not seem likely, as under the same conditions methylene blue is not reduced in 24 hours and there is no turbidity or smell of decomposition in the tube.   In 48 hours bacteria do appear and methylene blue is also reduced.   If we add chloroform, toluene, or thymol to the tubes of *Cypridina* extract to prevent the growth of bacteria, and allow them to stand 48 hours, upon admitting air the tube with chloroform gives no light but the tubes with toluene and thymol do give light although it is not so bright as if they were absent.   I believe that these substances have a destructive action on the reductases, most complete in the case of chloroform.

I have not been able to demonstrate that a *Cypridina* extract will reduce methylene blue or nitrates to nitrites either with or without the addition of acetaldehyde.   This may be due to the fact that oxyluciferin, which is also present, may be reduced more readily than either nitrates or methylene blue and so is reduced first.

Dubois[14] has described in *Pholas* a precursor of luciferin, which he calls proluciferin, which is converted into luciferin by another enzyme, coluciferase.   The proluciferin is not destroyed by boiling and the coluciferase will withstand a higher temperature than luciferase and may be freed of luciferase in this manner.   He cites an experiment[15] to prove the existence of proluciferin and coluciferase in *Pholas* but I have been unable to repeat this with *Cypridina*.   One might suppose that on allowing an extract of *Cypridina* (luciferase) to stand in absence of oxygen, some proluciferin, assuming this to be present, would be converted into luciferin which would give light if air was admitted.   But we can allow a boiled extract of *Cypridina* (containing no coluciferase) to stand with milk or muscle tissue suspensions

[13] This experiment may also be performed with *Pholas* luciferase with a similar result.

[14] Dubois, *Compt. rend. Soc. biol.*, 1907, lxii, 850; 1917, lxxx, 964.

[15] Dubois, *Compt. rend. Soc. biol.*, 1917, lxxx, 964.

in absence of oxygen and, upon admitting air and adding luciferase, obtain light.   As luciferase is only found in luminous animals it does not seem likely that a coluciferase would be widespread but we do know that a reducing enzyme occurs in milk and tissue extracts—is, in fact, widespread.   It seems more logical to interpret the above experiments as due to the reduction of an oxyluciferin to luciferin rather than the conversion of a proluciferin to luciferin.

Indeed, we can reduce oxyluciferin by means which do not involve the use of animal extracts and consequently are free from the objection that "coluciferase" may be responsible for the result, but which, nevertheless, are perfectly well known reducing methods. Perhaps the best of these is reduction by palladium black and sodium hypophosphite.   The latter is oxidized in presence of palladium and nascent hydrogen is set free.[16]   The nascent hydrogen reduces any easily reducible substance which may be present, such as methylene blue or oxyluciferin.   Oxyluciferin is not reduced by palladium alone or hypophosphite alone but methylene blue is reduced by palladium black alone.

If hydrogen sulfide is passed through a solution of methylene blue the dye is very quickly reduced and becomes colorless.   If the H_2S is driven off by boiling the colorless methylene blue solution, the blue color again returns on cooling.   Oxyluciferin can also be reduced to a certain extent by H_2S.   Sulfur dioxide or oxides of nitrogen (prepared by the action of HNO_3 on Cu) had no reducing action on either methylene blue or oxyluciferin.

Dilute acid favors the reduction of oxyluciferin.   If one saturates an oxyluciferin solution with CO_2 or adds a little dilute acetic acid and allows the solution to stand for 24 hours, a certain amount of reduction will occur.   No reduction occurs if the solution is saturated with pure hydrogen and allowed to stand 24 hours.   If one adds some Mg powder to oxyluciferin and then dilute acetic acid in successive additions as the acetic acid is used up in formation of Mg acetate, the oxyluciferin will be reduced relatively quickly.   Nascent hydrogen is produced in the reaction and is no doubt the active reducing agent, while the acid accelerates the reduction.   Soured milk also has

[16] Bach, *Ber. chem. Ges.*, 1909, xlii, 4463.

quite a marked reducing action. Acid thus favors reduction and hinders oxidation, while alkali favors oxidation and hinders reduction of the oxyluciferin.

While I have not studied the properties of oxyluciferin so fully as those of luciferin, as far as I can judge both substances give the same general reactions and possess identical properties. If we make a concentrated hot water extract of *Cypridina*, it will contain all the substances of the animal soluble in hot water and not coagulated by heat and may be spoken of as crude luciferin solution. If air is bubbled through this solution for some time, all the luciferin is oxidized and it may then be spoken of as crude oxyluciferin solution. Both crude luciferin and crude oxyluciferin solution are yellow in color, but I do not believe that either luciferin or oxyluciferin is yellow in color because an ether or benzene extract of *Cypridina* is also yellow although luciferase, luciferin, and oxyluciferin are all insoluble in ether and benzene. The yellow pigment which can be observed to make up part of the luminous gland of *Cypridina* is not luciferin or luciferase. It may be a pigment related to urochrome.

When tests are applied and precipitating reagents are added to crude luciferin and crude oxyluciferin solution they give identical results in each case. Thus if crude luciferin is saturated with $(NH_4)_2SO_4$ or $MgSO_4$, a flocculent precipitate forms which may be demonstrated to contain most of the luciferin. Oxyluciferin solution also gives flocculent precipitates on saturation with $(NH_4)_2SO_4$ and $MgSO_4$ and these contain most of the oxyluciferin. To demonstrate this the precipitates, after washing, are dissolved in a small amount of water mixed with fresh milk (or frog muscle suspension) and allowed to stand in a stoppered tube for 24 hours. If any oxyluciferin is present it will be reduced to luciferin and give light when luciferase is added. One-half saturation with $(NH_4)_2SO_4$ or $MgSO_4$ or saturation with NaCl salts out no material from either crude luciferin or oxyluciferin solution. Picric acid gives no precipitate but only an opalescence in both cases. In a similar manner it may be shown that most of the oxyluciferin is precipitated by phosphotungstic acid but not by acetic acid or CO_2, in this respect also agreeing with the behavior of luciferin. Like luciferin the oxyluciferin will pass porcelain filters, dialyze through parchment or collodion membranes, is soluble

in absolute alcohol but not in ether or benzene, and is undigested by salivary diastase, pepsin HCl, Merck's pancreatin in neutral solution, and erepsin. The salivary diastase and the pancreatin (containing amylopsin, trypsin, and lipase) were allowed to digest for 4 days at 38°C. without showing any evidence of digestive action. Oxyluciferin is partially but not completely precipitated by basic lead acetate and tannic acid.

As luciferin is so easily oxidizable a substance, we should expect to find that it will reduce just as glucose will reduce. However, a concentrated solution of luciferin has no reducing action on Fehling's (alkaline Cu), Barfoed's (acid Cu), Nylander's (alkaline Bi), or Knapp's (alkaline Hg) reagent. Glucose will reduce methylene blue in alkaline (not in neutral) solution but luciferin will not reduce methylene blue in alkaline or neutral solution. It would seem, then, that luciferin must contain no aldehyde group. If so, we should expect to obtain reduction of some of the above reagents. Just what group is concerned in the oxidation is unknown at the present time and speculation regarding it in the absence of more experimental data can be of little value.

THE PHOTIC SENSITIVITY OF CIONA INTESTINALIS.

By SELIG HECHT.

(From the Physiological Laboratory, College of Medicine, Creighton University, Omaha.)

I. Preliminary Statement.

The sensitivity of *Ciona intestinalis* to light furnishes an unusual opportunity for analysis of the receptor process occurring in the sense organ. This is because a sudden increase in illumination produces a vigorous and clear cut response on the part of the animal. Such a reaction to a source of stimulation, which in itself may be measured accurately and controlled with comparative ease, makes the sensory behavior of *Ciona* amenable to a strict, quantitative treatment. My experiments were therefore concerned, first, with the localization of the photic sense organs, and second, with variations in the magnitude of the stimulating agent under conditions calculated to throw light on the nature of the sensory process.

The species used is identical with the *Ciona intestinalis* common in the Mediterranean Sea and along the European coast (Ritter and Forsyth, 1917). At San Diego, California, it is to be found in enormous numbers attached to piles, and forming thick carpets on the under side of floats. In fact, the hundreds of individuals employed were all taken from a single float in San Diego Bay.[1]

II. Photic Sense Organs.

1. *Ciona* possesses two qualitatively different means of reacting to light. In one case the response is a general one, involving the organism as a whole. In the other the activity is confined to the lo-

---

cality which receives the stimulation. I shall describe the latter first because of its comparative insignificance in the experiments to be presented.

The body of *Ciona* may be roughly divided into three parts: an anterior region occupied by the siphons and extending below the intersiphonal neural mass; a posterior region containing the viscera and reproductive organs; and a middle region composed in the main, of the translucent branchial sac. The posterior region is practically insensitive to light. The anterior region, *with the notable exception of the area around the neural mass*, is similarly insensitive. The middle portion, however, is quite responsive to photic stimulation.

Light focussed on different parts of this area resulted in a purely local contraction of the circular and longitudinal muscle fibers. When a part of this area on one side was exposed to light, the animal curved on its long axis toward the exposed side. By repeatedly exposing first one side and then the other to this local stimulation, the animal could be made to curve back and forth at will. A similar motion was secured by alternately exposing the dorsal and ventral edges of an animal. As a result, it would swing back and forth, dorsally and ventrally, subject to the frequency of stimulation.

This sensitivity persists in its original strength after the intersiphonal ganglion has been removed, showing its independence of central control. That we are not dealing here with a case of direct stimulation of the muscular tissue (Parker, 1916) is shown by its behavior under chloretone anesthesia. When a *Ciona* is placed in a solution of chloretone in sea water it loses this local photic sensitivity simultaneously with its irritability to touch and to another type of light stimulus which will presently be shown to be undoubtedly mediated by a nervous mechanism. In all probability there is present here a neuromuscular complex similar to the nerve net of lower invertebrates, which is sensitive to light. Evidence for the existence of such a nerve net has already been presented by Jordan (1908) on the basis of his work on the mechanical stimulation of *Ciona*.

2. Under ordinary conditions of experimentation, this sensitivity and its local response are entirely masked by the other, more general, reaction to light. When a *Ciona* as a whole is exposed to a source of illumination, it reacts by a vigorous retraction of its siphons (Nagel,

1896). If the intensity of the stimulus is great, this may be accompanied by a shortening of the animal on its long axis. This retraction reflex has already become familiar due to the work of Loeb (1902) and of Jordan (1908) among others (see Hecht, 1918, b) especially in its relation to the removal of the ganglion of *Ciona*.

It is the response of *Ciona* by means of the retracting reflex that forms the basis of the experiments which follow.

3. The orange or red pigment spots on the rims of the siphons are a prominent characteristic of ascidians. *Ciona* possesses eight on the oral rim and six on the atrial. These pigmented masses have been called ocelli on the assumption that they are the photoreceptors of *Ciona*.

Experiments designed to ascertain the location of the photic sense organs showed that the anterior pigment spots were not sensitive to light. A beam of light focussed upon them failed to produce the characteristic reaction. Moreover, their complete removal in no way interfered with the sensitivity of the animal. The protocol of Animal 25.1 gives the details of such an experiment.

Animal 25.1.

Time.
11.00 In dark room.
2.25 Exposed to light, 120 candle power, reaction time........... 3.5 sec.
2.35   "   "   " 120 "   "   "   " .... 3.3 "
2.44   "   "   " 120 "   "   "   " .... 3.3 "
2.50 Dirty translucent test of animal removed.
4.30 Exposed to light, reaction time...................... 1.7 sec.
4.37   "   "   "   "   " .... 1.4 "
4.44   "   "   "   "   " .... 2.2 "
4.50 Rims cut away to remove the ocelli of both siphons.
7.41 Exposed to light, reaction time...................... 1.4 sec.
7.49   "   "   "   "   " .... 1.6 "
8.00   "   "   "   "   " .... 2.0 "
10.00   "   "   "   "   " .... 1.9 "

Of fourteen animals operated in this way, two were killed accidentally. The remaining twelve all gave the same results as the one presented in detail. It is clear, therefore, that these pigment spots are not photoreceptive organs (cf. Hecht, 1918, b).

It has already been shown that although the general body surface is sensitive to light, the resulting activity is a purely local one. Gen-

eral body sensitivity could not therefore account for the retraction reflex. By exploring the body with a small beam of light, I found that the seat of this photic sensitivity was in the small area occupied by the intersiphonal ganglion and its related structures. When light was focussed on this spot alone the reaction given by *Ciona* was in all particulars the same as when the animal as a whole was illuminated. No other region of the body possessed this property. As an example of many similar experiments the record of Animal 30.5 is given.

Animal 30.5.

Time.	Part stimulated.	Reaction time.
		sec.
11.40	Entire body.	13.1
11.45	Oral ocelli.	No reaction.
11.50	Ganglion region.	5.0
11.58	" "	5.2
1.57	" "	5.8
2.08	Atrial ocelli.	No reaction.
8.15	Ganglion region.	4.8

It will be noticed that the reaction time to stimulation of the ganglionic region is less than for the exposure of the entire body. This is because the light used for spotting the ganglion region was concentrated to a small area by means of a lens. It will be shown presently that the reaction time varies inversely as the intensity of the light.

It is difficult to say which of the several organs composing the neural mass contains the photoreceptors. Experiments with the opaque *Ascidia atra* of Bermuda (Hecht, 1918, *b*) have shown that its photic sensitivity may be demonstrated only by stimulating the dorsal wall of the *inside* of the oral siphon below the ring of tentacles. This corresponds to the position of the dorsal tubercle, a structure which, in *Ciona*, is closely affiliated with the ganglion. However, though I performed many operations involving the removal of the entire neural mass, as well as only portions of it, the results were of rather doubtful interpretation.

The difficulty probably lies in the fact that a disturbance in the integrity of the neural complex interferes with the conduction of im-

pulses in this region (Fröhlich, 1903). Consequently it was impossible to make a finer localization of the sense organs responsible for the general photic reaction. The presence of the sense organs in the definitely circumscribed region of the neural mass, however, is decisive and clear cut. In a medium sized individual this structure occupies a volume of about 1 cc. or less. In addition it should be pointed out that this area of sensitivity is colorless and contains no apparent pigment of any kind (cf. Wenrich, 1916, p. 306). For the most part the structures are transparent; the ganglionic mass itself, however, is glistening white.

III. Analysis of Reaction Time.

1. The appearance of the retraction reflex as a result of photic stimulation possesses one very important property. This is the presence of a reaction time representing the interval from the beginning of the exposure to light up to the moment when the retraction takes place. This interval may be measured accurately with a stop-watch, first, because it is of rather long duration, and second, because the appearance of the retraction reflex is sudden and clear cut.

The reaction time of any individual *Ciona* to a given intensity of light shows almost no variations from a constant quantity. In Table I are given the reaction times of three animals to the same intensity of light taken at different times of the day. When not exposed to the light, the animals were all kept in a dark room. From these figures it will be seen that the reaction time represents a quantity which may be reproduced under a given set of conditions.

TABLE I.

Light Intensity, 4,880 Candle Meters.

Animal 3.1		Animal 3.2		Animal 3.3	
Time.	Reaction time.	Time.	Reaction time.	Time.	Reaction time.
	sec.		sec.		sec.
1.56	2.8	1.57	1.9	1.59	3.6
4.27	3.2	4.28	2.1	4.30	3.9
9.12	2.9	9.14	2.0	9.15	3.5

2. Before this study had proceeded very far it became clear that the reaction time could not be reduced below approximately 1.5 seconds. No matter how intense the stimulating light, this interval was always maintained. To explain this, I adopted the provisional hypothesis that the reaction time was not a simple interval but that it contained at least two components. This indeed, was soon found to be true, when experiments were made to determine how much of the reaction time was actually devoted to the reception of the photic stimulus and how much was concerned with a real latent period.

By exposing an animal for a shorter time than its reaction time, an interval was found during which it was not necessary for *Ciona* to be illuminated. At the end of this interval it reacted as usual although at the time of reaction, like the hydroids used by Loeb and Wasteneys (1917), they were in the dark. The reaction time is therefore composed of two parts. The first is a sensitization period during which *Ciona* must be exposed to light. The second is a latent period during which *Ciona* need not be illuminated in order to react at the end of the period. With a little practice the sensitization period could be regulated to within 0.2 second. An example is given by Experiment 8.6.

Experiment 8.6.   Light Intensity, 743 Candle Meters.

Time.	Time exposed.	Reaction time.
	sec.	*sec.*
8.34	3.6	3.6
9.18	3.5	3.5
10.00	2.0	3.5
10.35	1.0	No reaction.
10.58	1.5	" "
11.32	1.5	" "
12.02	2.0	3.7
1.19	2.0	3.4

Result: Sensitization period = 2.0.   Latent period = 1.5.

With the duration of the sensitization period we shall be concerned later, because it varied with the intensity of the light. The latent period, however, showed itself to be a constant quantity under considerable variations in intensity. A series of 53 determinations of

the latent period of nineteen animals gave 1.76 seconds as the average duration of this portion of the reaction time.

3. The latent period must include the time for transmission of the stimulus and kindred phenomena. There is evidence to show, however, that a great portion of it is concerned with a process occurring in the sense organ itself, as is shown in Experiment 9.4.

Experiment 9.4. Light Intensity 4,880 Candle Meters.

Time.	Time exposed.	Reaction time.
	sec.	*sec.*
1.31	2.4	2.4
2.31	2.3	2.3
2.53	0.2	No reaction.
3.24	0.5	7.6
3.41	0.8	3.6
3.47	1.0	2.8
4.22	1.0	2.3
4.43	2.1	2.1

It will be seen that, within limits, the shorter the exposure time (sensitization period) the longer the reaction time, and consequently the latent period. The latent period in the main must therefore take place in a locality where it may be immediately influenced by the amount of light energy received. This would indicate that it is the outward manifestation of the duration of a process within the sense organ itself.

4. The existence of a latent period of nearly 2 seconds is hardly to be considered as an advantage to the life of the species. The ordinary daylight of southern California is deleterious to *Ciona*. The animals die very soon in an aquarium in diffuse daylight. When placed near a window they contract and perish in a day, whereas in a dimly lighted room or in the dark they remain in excellent condition for a week or more. They are normally found on the under side of floats.

The comparatively long delay in responding after a sufficient stimulus has been received cannot thus be of assistance to the animal in its response to a noxious stimulus. It must therefore be attributed to the duration of a chemically necessary process occurring as suggested, in the sense organ.

IV. Effect of Intensity.

1. In order to study the effect of different intensities of light on the duration of the sensitization period, a single source of light was used. This was a Mazda lamp of 120 candle power. By keeping it at different fixed distances from the animal, six intensities were secured, as given in Table II.

Ten animals varying in size from 3.5 to 10.5 cm. were used for this experiment. It may be said at once that no difference in sensitivity was found for individuals of different size. The reaction time of each animal to each of the six intensities of light was determined three times; thus, eighteen observations were made on each animal.

TABLE II.

1	2	3	4
Intensity (*I*).	Reaction time.	Sensitization period (*t*).	*I* × *t*
candle meters	*sec.*	*sec.*	
521	11.25	9.49	4,944
743	8.89	7.13	5,298
1,180	5.98	4.22	4,980
2,080	3.59	1.83	3,806
4,880	2.72	0.96	4,685
9,920	2.24	0.48	4,762
Average...			4,746

The determinations were purposely scattered over a period of 12 hours so that any fatigue effects would be avoided. In Table II, Column 2, are given the average values for the reaction times of the ten animals. Each figure represents an average of 30 determinations. In addition the data are plotted in Curve A of Fig. 1. From both these sources it is apparent that the reaction time varies inversely as the intensity. In Column 3 are given the values for the sensitization periods corresponding to the different reaction times. These were calculated by subtracting the duration of the latent period, 1.76 seconds, from the reaction time values.

2. On the supposition that the sensitivity of *Ciona* depends upon a photochemical reaction, the relation between the intensity of the

light and the sensitization period should be expressed by the reciprocity law as found by Bunsen and Roscoe (1862). This states that for a given photochemical effect (E) a definite quantity of energy is required, and this same result is obtained whether the intensity (I) is high and the exposure (t) short or conversely. In other words,

$$E = k \cdot I \cdot t \quad (1) .$$

The applicability of this law has been demonstrated by Blaauw (1909) and by Fröschel (1909) for the photic reactions of some plants, and by Loeb and his coworkers for the orientation of several species of animals. In *Ciona*, its existence would be demonstrated if the product of the intensity and the sensitization period were a constant quantity. In Column 4 of Table II are given the values obtained. It will be seen that the figures agree well with one another and with the average value.

The matter may be illustrated graphically. The equation representing the Bunsen-Roscoe law may be written

$$\frac{E}{t} = k \cdot I \quad (2)$$

In this form, it represents the equation of a straight line. Since, in *Ciona*, E is constant, $\frac{E}{t}$ may be represented by the reciprocal of the sensitization period, and the relation between this quantity and the intensity should be represented by a straight line. Such indeed is the case, as shown by the position of the points in Curve B of Fig. 1.

3. In terms of the processes that go on in the sense organs, the applicability of the reciprocity law means that during the sensitization period the light must form a constant quantity of a substance before it can produce its stimulating effect. Undoubtedly this involves the conversion of a photosensitive substance into something else which acts as an "inner stimulus." Many such photosensitive substances are well known and have been studied with considerable care (Sheppard, 1914).

With a more precise presentation of the dynamics of the photochemical reaction in *Ciona* we shall deal in a following section. At present, however, I wish to emphasize a corollary to the fact that the

photic reaction proceeds according to the Bunsen-Roscoe rule as indicated in equation (1). It is, that, with a given intensity of illumination, the sensitization period indicates the quantity of stimulus received by the animal. The photochemical effect (E) will vary with the duration of the exposure (t) when the intensity (I) is constant.
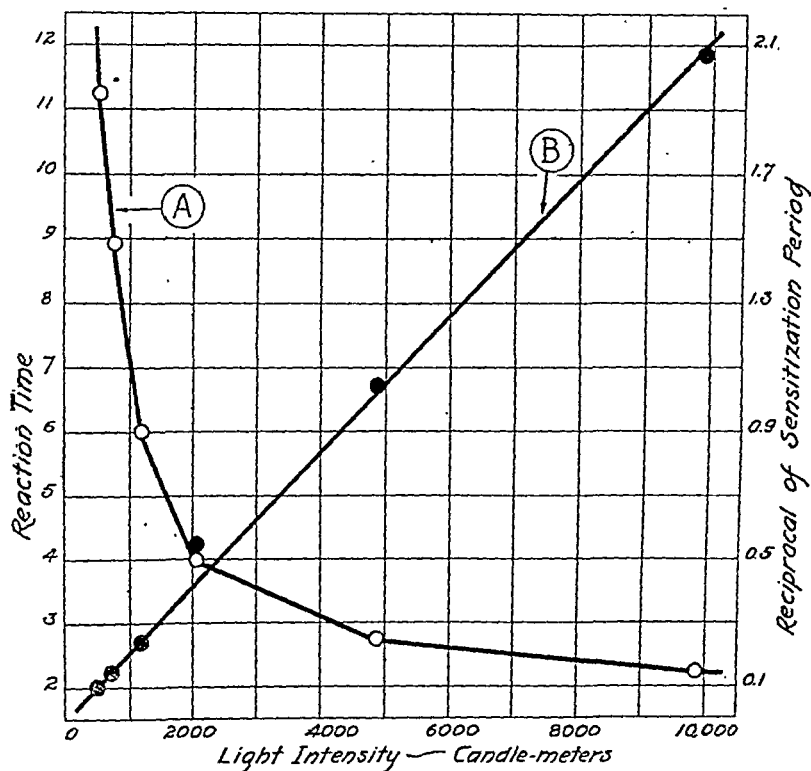
FIG. 1. Curve A gives relationship between intensity of light and reaction time of *Ciona*. This curve is an hyperbola as is shown by Curve B representing the relation between the reciprocal of the sensitization period and intensity.

The reaction time at any moment may therefore be used as a measure of the amount of photochemical effect necessary in order to act as a stimulus in the sense organ. In this way we may investigate the sensitivity of *Ciona* under different conditions in order to gain a clearer insight into the photochemical process which underlies it.

V. Dark Adaptation.

1. Under ordinary circumstances, *Ciona* remains expanded in diffuse light. The stream of sea water flows steadily through its branchial sac except for certain rhythmic interruptions presented in detail elsewhere (Hecht, 1918, *a*). Similarly, the animal becomes non-responsive after a time when placed in a lower or a higher intensity of illumination. After such adaptation it fails to react to a decrease in the intensity of the illumination but responds vigorously to an increase. On remaining for some time in the dark—becoming "dark adapted"—it is sensitive to the light of an ordinary Mazda lamp.
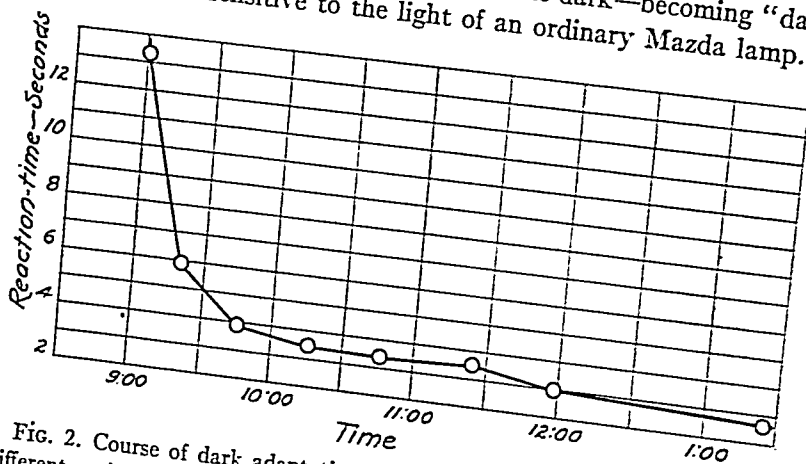
Fig. 2. Course of dark adaptation of *Ciona* represented by reaction time at different periods of sojourn in dark room.

The process of dark adaptation was followed in some detail. Animals which had been in the light for a few hours were removed to the dark room and their responses to light noted at regular intervals. A constant intensity was used, so that any variations in the reaction time would serve to indicate corresponding variations in the quantity of stimulating agent required to cause a reaction.

The results were regular and fairly uniform. The process of dark adaptation required at least 2 hours; usually more. At first the reaction time was long, then it shortened rapidly, then more slowly, and finally at the end of 2 or more hours it reached a constant minimum. A good example of what occurred is given by Fig. 2. The points on

the curve represent the averages of the reaction time of two individuals which were used in this particular experiment.

At any given point, the duration of the reaction time (really of the sensitization period) measures the quantity of light energy required by the photochemical system to produce a response of *Ciona*. In terms of the Bunsen-Roscoe law this, in addition, measures the photochemical effect, and consequently the amount of photosensitive substance which must be changed before the retraction reflex can be elicited. The curve in Fig. 2 shows this quantity to be at first large, then smaller and smaller. At the end of the process of dark adaptation it becomes constant at its minimal value. The interpretation of these changes will be considered in the photochemical system now to be presented.

VI. *Nature of Photochemical System.*

1. The decomposition of a photosensitive material by light presupposes the formation of such a substance and its deposition within the sense organ. The nature and dynamics of the formation and decomposition of the photosensitive material must be consistent with the four phases of the photic behavior of *Ciona*. First, it must explain the ability of *Ciona* to come to sensory equilibrium in any intensity of light. Second, it must be clear why under given conditions the reaction time to a definite increase of illumination is constant. Third, the process must be consistent with the Bunsen-Roscoe rule for the relation between intensity and sensitization period. Fourth, it must indicate the basis of the regular change in the reaction time that occurs during dark adaptation.

2. The simplest assumption which will account for all the phenomena observed is that we are dealing here with a reversible chemical reaction. A photosensitive material (S) is formed from its precursor (P), the reaction being of the first order and following the ordinary laws of such processes. It may be of a higher order, but the assumption is made for the sake of simplicity. The action of light upon the photosensitive material (S) is to change it back into its precursor (P). In accordance with current photochemical knowledge such a reaction will follow the Bunsen-Roscoe rule. A reaction which in many respects resembles the system suggested here, is the conversion of

anthracene into dianthracene by light, and its reversal in the dark (Sheppard, 1914, p. 214).

Let us consider the reaction which occurs normally, and of course in the dark, and which is supposed to regenerate the photosensitive material from its precursor ($P \rightarrow S$). The curve in Fig. 3 indicates the process on the basis of the ordinary velocity curves for chemical reactions. The formation of the photosensitive material (ordinates at the right) proceeds at first rapidly, then it loses speed gradually, and finally it reaches a point of equilibrium which represents a definite ratio between the concentration of the sensitive material and its precursor. The important thing to be noticed in the curve is the change
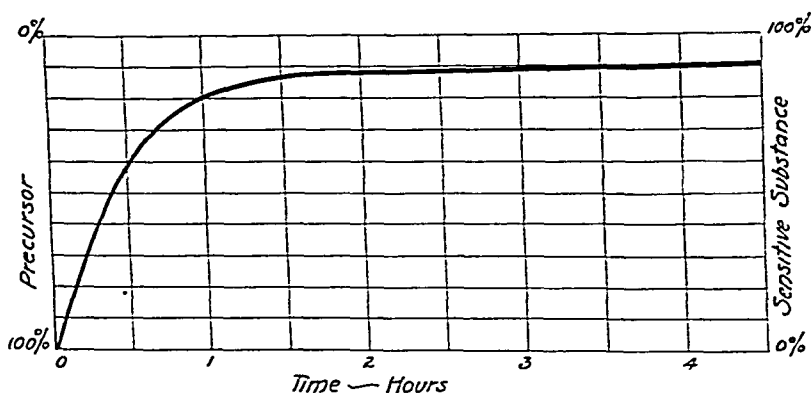
FIG. 3. Hypothetical curve of reaction $P \rightarrow S$ based on data of Fig. 2.

in the quantity of precursor (ordinates to the left) during the reaction. At first the amount of this substance is large, then it decreases rapidly, then more slowly, and finally it reaches a constant minimum. On comparing this rate of change with the one undergone by the reaction time during dark adaptation it is seen that the two run quite parallel. It has been assumed that the action of the light during the sensitization period causes the photosensitive material to be changed into its precursor. This means that the variation in the amount of the precursor formed at the successive reactions during dark adaptation is in general similar to the change in the amount of precursor still unused in the chemical reaction. From the relationship between these two variables we must conclude that, in order to act as a stimu-

lus in the sense organ, the quantity of precursor formed by the stimulating light bears a definite ratio to the amount already present. As a matter of fact the curve in Fig. 3 is not a theoretical one, but was actually constructed from the data of Fig. 2, on the assumption that the ratio between the precursor formed and the precursor already present is as 1: 10. The fact that it resembles the ordinary velocity curves for chemical reactions strengthens the force of the above explanation.

The necessity for the acquisition of a constant ratio between precursor formed and precursor present, before stimulation can occur, is essentially the requirement of the Weber-Fechner concept. This states that the amount of stimulus necessary to produce a perceptible increase in sensory effect represents a constant fraction of the quantity of stimulus that has previously been applied. Waller (1895) has already shown that the Weber-Fechner law is dependent upon the processes that go on in the sense organ and not in the transmitting mechanism or in the central nervous system.

3. It is necessary now to show how the photochemical system suggested above can account for the sensory phenomena in *Ciona*. The investigations of such reversible reactions as proposed here, in particular the work of Luther and Weigert (1905) on the polymerization of anthracene by ultra-violet light, give us some idea of the processes occurring in our reaction system:

$$P \underset{\text{light}}{\overset{\text{"dark"}}{\rightleftarrows}} S$$
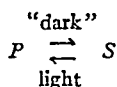
The stable condition is that of the sensitive material S in the dark. Starting with the precursor P in the dark, it changes spontaneously to S. Light converts S back into P. However, because the reverse change, $P \rightarrow S$, is independent of light, it will be resumed as soon as some P has been formed by the light. Moreover, the more precursor (P) that is formed by the light, the greater will be the rate at which the photosensitive substance will be formed. This follows from the ordinary mass action principle. At a given intensity of illumination the two reactions will proceed in opposite directions at an equal rate, and a "stationary condition" simulating a chemical

equilibrium will be reached, in which the quantity of precursor will be constant. This will happen at any intensity of light no matter from which direction it is approached.

Constancy in the quantity of precursor means no stimulation of *Ciona*. We have thus a mechanism which possesses the first requirement for an explanation of the photosensitivity of *Ciona*. This, it will be recalled, is the ability of the animal to come into sensory equilibrium at any intensity of illumination. An explanation of the second characteristic of the sensitivity follows from this at once. In order to act as a stimulus the increase in illumination must be maintained for such a time as to produce a definite fraction of the precursor present. This is constant; therefore, the reaction time will be constant. The third requirement is also easily deduced. The quantity of precursor required for stimulation is constant. Since the reaction $P \leftarrow S$ follows the reciprocity law, it will make no difference whether the intensity is low and the sensitization period long or *vice versa*. The variation in the reaction time during dark adaptation has already been dealt with to show that it depends on the suggested photochemical system. The latter therefore completely fulfills our demands for an explanation of the photic responses of *Ciona*.

4. It should be pointed out that the proposed chemical system is the simplest, but not the only hypothesis capable of explaining the sensory phenomena observed in *Ciona*. There are two reactions required: one, a "light" reaction, and the other, a "dark" reaction. It has been assumed that these are the two phases of a reversible reaction system. A somewhat more complex, yet essentially similar hypothesis is to consider the two reactions as forming a catenary system.

According to this alternative suggestion, a photosensitive substance S is decomposed, not into its precursor but into another substance DS. This is the "light" reaction. The decomposition product DS then becomes converted into an inert material M. This is the "dark" reaction. Thus

$$S \underset{\text{light}}{\rightarrow} DS \quad (1) \qquad \text{and} \qquad DS \underset{\text{"dark"}}{\rightarrow} M \quad (2)$$
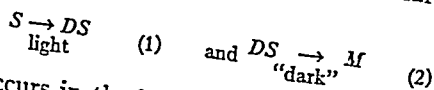
Reaction (1) occurs in the light and follows the Bunsen-Roscoe law. The decomposition product (DS) is the stimulating substance, and in

order to act as a stimulus the amount of it to be formed by the light must bear a constant ratio to the amount of DS already present in the system.   Reaction ·(2) removes the decomposition product from the reaction system.   It possesses the same velocity and properties as the "dark" phase of the reversible reaction system presented before.   Its occurrence is represented by the process of dark adaptation shown in Fig. 2, and its course is given by the curve in Fig. 3 in which P represents DS and S represents M.   ·

Which of the two suggested photochemical systems comes nearer the truth future work must decide.   The present data may be explained in terms of either.   The simpler one is therefore provisionally adopted.

VII. Repeated Stimulation.

1. There are several predictions which may be made on the basis of the hypothetical chemical system which has been proposed.   My stay at La Jolla, however, limited me to an investigation of but one of these.

We have seen that the "dark" reaction $(P \rightarrow S)$ is a comparatively slow one, requiring several hours to reach an equilibrium.   Therefore, if a thoroughly dark-adapted *Ciona* is stimulated by light at short intervals, say every minute, practically no new photosensitive material will be regenerated during a period of about 10 minutes. The quantity of precursor however will be continually increased, depending upon the duration of the exposure to light.   This follows from the Bunsen-Roscoe rule which has been shown to apply to *Ciona*. The quantity of precursor necessary to stimulate must be a constant fraction of the amount of precursor already present.   Therefore, during such a series of regular stimulations, the amount of precursor required must be larger at each succeeding reaction.   The rate of increase in the amount of precursor will be logarithmic, each quantity representing the same fraction of the sum of the quantities that have been formed before.   The sensitization period measures the quantity of precursor formed at each reaction.   Therefore, in such a set of repeated stimulations, the sensitization periods should always bear a definite ratio to the sum of the sensitization periods that have preceded it.

All the experiments made to test this prediction verified it with amazing unanimity. *Cionas* which had been in the dark for several hours were exposed to a light of 2,000 candle meters at regular intervals of 1 minute and the reaction time at each exposure noted. The results of four experiments are given in Figs. 4 and 5.

The upper curve in Fig. 4 represents the actual data, the moments of stimulation being the ordinates and the sensitization periods the abscissæ (upper scale). The curve has the usual Weber-Fechner appearance, its logarithmic nature being shown in the lower curve of the
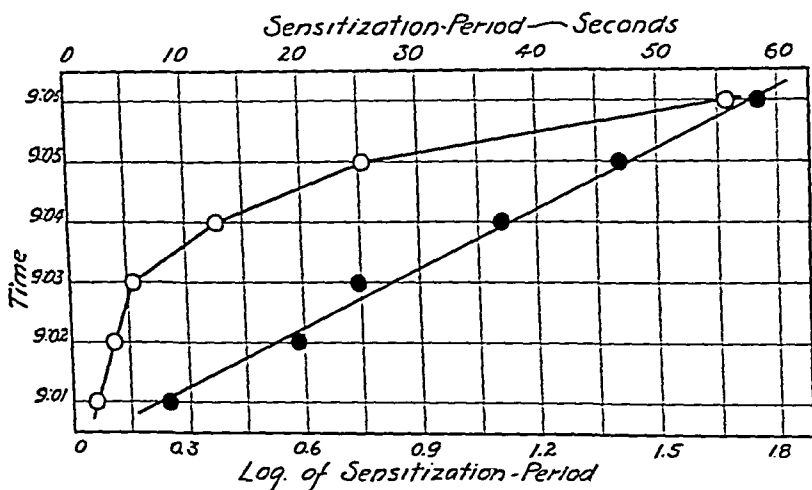
FIG. 4. Upper curve gives sensitization periods of a *Ciona* during regularly repeated stimulation at minute intervals. Its logarithmic nature is shown by the lower curve which gives the logarithms of the same sensitization periods.

same figure. The lower curve has the same ordinates, but the abscissæ are the logarithms of the sensitization periods (lower scale). That the points form a straight line is undoubted.

In Fig. 5 are given the data for three other animals. The logarithms of the sensitization periods are plotted as before, and in each case the points very clearly form a straight line.

This series of experiments therefore is entirely consistent with the three cardinal features of the hypothetical chemical system proposed as an explanation for the photic sensitivity of *Ciona*. These are, first,

that the "dark" reaction of sensitive material regeneration is comparatively slow; second, that the "light" reaction changes the sensitive material into its precursor according to the Bunsen-Roscoe law; and third, that in order to act as a stimulus, the light must form such a quantity of precursor that it will bear a definite ratio to the amount of precursor already present.

2. There is a phase of these data on regularly repeated stimulation to which attention has already been called (Hecht, 1918, *c*) and which must be emphasized. The individual experiments were ended when the animal failed to react to the light. The course of an experiment
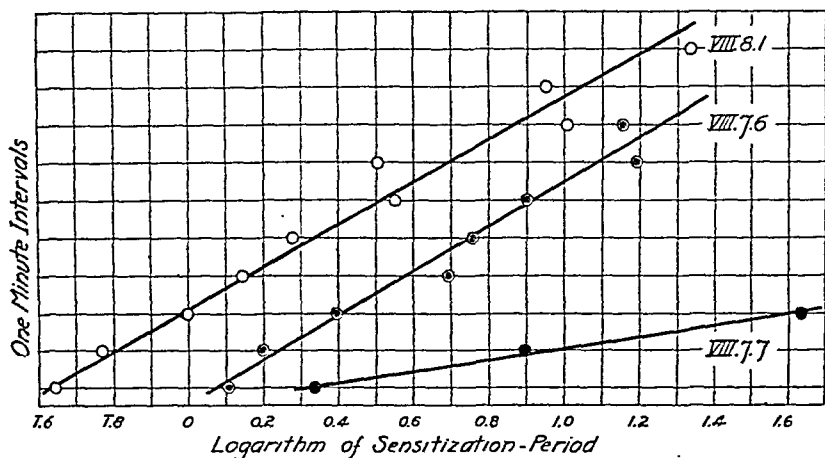
Fig. 5. Data of three other individuals plotted similarly to lower curve of Fig. 4.

was therefore as follows. The reaction time to the same intensity became larger and larger and finally the animal ceased to react,—because "adapted" to the stimulus. This is the kind of evidence which has been used to prove the existence of a "higher behavior" in lower animals (Kinoshita, 1910). According to this line of reasoning, the increased reaction time represents a process of learning, and the complete cessation at the end a condition of "indifference" to the stimulus, or a state of adaptation.

I hope that the experiments presented in this communication will show the futility of such merely verbal explanation. From the data

given here it seems clear that the entire process takes place in the sense organs themselves. The attainment of a condition of sensory equilibrium of *Ciona* depends upon the existence of a "stationary condition" of the reversible reaction system responsible for its photosensitivity. Dark adaptation represents such a sensory equilibrium when the "light reaction" is completely removed. Rapid adaptation to light is essentially the reverse process, in which the "dark" reaction is practically absent, and the "light" reaction is in full swing.

In these experiments there is no need either for the postulation of a learning process or for the presence of a "higher behavior" in order to rationalize the results obtained.

SUMMARY.

1. *Ciona* possesses two means of responding to an increase in the intensity of illumination. One is by means of a local reaction; the other is by a retraction reflex of the body as a whole.

2. The "ocelli" are not photoreceptors. The photosensitive area is in the intersiphonal region containing the neural mass. This area contains no pigment.

3. The reaction time to light is composed of a sensitization period during which *Ciona* must be exposed to the light, and of a latent period during which it need not be illuminated in order to react to the stimulus received during the sensitization period.

4. The duration of the reaction time varies inversely as the intensity. Analysis shows the latent period to be constant. The relation between the sensitization period and the intensity follows the Bunsen-Roscoe rule.

5. During dark adaptation the reaction time is at first large, then it decreases until a constant minimum is reached.

6. A photochemical system consisting of a reversible reaction is suggested in order to account for the phenomena observed. This system includes a photosensitive substance and its precursor, the dynamics of the reaction following closely the peculiarities of the photosensitivity of *Ciona*.

7. It is shown that in order to produce a reaction, a constant ratio must be reached between the amount of sensitive substance broken down by the stimulus and the amount previously broken down.

8. From the chemical system suggested certain experimental pre-

dictions were made. The actual experiments verified these predictions exactly.

9. The results obtained with regularly repeated stimulation not only fail to show any basis for a learning process or for the presence of a "higher behavior," but follow the requirements of the photochemical system suggested before.

BIBLIOGRAPHY.

Blaauw, A. H., Die Perception des Lichtes, *Rec. Trav. Bot. Néerlandais*, 1909, ii–iv, 209.

Bunsen, R., and Roscoe, H., Photochemische Untersuchungen. VI, *Ann. Physik.*, 1862, cxvii, 529.

Fröhlich, A., Beitrag zur Frage der Bedeutung des Centralganglions bei *Ciona intestinalis*, *Arch. ges. Physiol.*, 1903, xcv, 609.

Fröschel, P., Untersuchung über die heliotropische Präsentationszeit. II, *Sitzungsb. Wien. Akad.*, 1909, cxviii, 1247.

Hecht, S., (*a*) The physiology of *Ascidia atra* Lesueur, I. General physiology, *J. Exp. Zool.*, 1918, xxv, 229; (*b*) II. Sensory physiology, *ibid.*, 261; (*c*) Adaptation in the photosensitivity of *Ciona intestinalis*, *Science*, 1918, xlviii, 198.

Jordan, H., Über reflexarme Tiere, Z. allg. Physiol., 1908, vii, 86.

Kinoshita, T., Über den Einfluss mehrerer aufeinanderfolgender wirksamer Reize auf den Ablauf der Reaktionsbewegungen bei Wirbellosen, I. Versuche an Tunicaten, *Arch. ges. Physiol.*, 1910, cxxxiv, 501.

Loeb, J., Comparative physiology of the brain and comparative psychology, New York, 1902.

Loeb, J., and Wasteneys, H., A reexamination of the applicability of the Bunsen-Roscoe law to the phenomena of animal heliotropism, *J. Exp. Zool.*, 1917, xxii, 187.

Loeb, J., and Northrop, J. H., Heliotropic animals as photometers on the basis of the validity of the Bunsen-Roscoe law for heliotropic reactions, *Proc. Nat. Acad. Sc.*, 1917, iii, 539.

Luther, R., and Weigert, F., Über umkehrbare photochemische Reaktionen in homogenen Systeme. Anthracen und Dianthracen, *Z. physik. Chem.*, 1905, li, 297.

Nagel, W. A., Der Lichtsinn augenloser Tiere, Jena, 1896.

Parker, G. H., The effector systems of actinians, *J. Exp. Zool.*, 1916, xxi, 461.

Ritter, W. E., and Forsyth, R. A., Ascidians of the littoral zone of Southern California, *Univ. California Publications, Zool.*, 1917, xvi, 439.

Sheppard, S. E., Photochemistry, New York, 1914.

Waller, A. D., Points relating to the Weber-Fechner law; retina; muscle; nerve, *Brain*, 1895, xviii, 200.

Wenrich, D. H., Notes on the reactions of bivalve mollusks to changes in light intensity: image formation in pecten, *J. Animal Behavior*, 1916, vi, 297.

AN INDICATOR METHOD OF MEASURING THE CONSUMPTION OF OXYGEN.

By W. J. V. OSTERHOUT.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

The study of respiration has been greatly facilitated by using indicators to measure the production of carbon dioxide. It is desirable to employ similar methods for estimating the amount of oxygen consumed in respiration. With this in view the writer has investigated a number of substances, some of which appear to give promising results. The purpose of the present note is to describe very briefly the use of *Limulus* blood for this purpose.

The blood of *Limulus* (and of a number of other invertebrates) when shaken up with air absorbs oxygen and turns blue, but on standing is reduced and in consequence becomes decolorized. It has been pointed out by Alsberg[1] and by Harvey[2] that the decolorization is due (in large part at least) to the activity of microorganisms. It seemed to the writer that this might be utilized to measure the rate of consumption of oxygen in respiration.

The procedure adopted is as follows: Large horseshoe crabs are taken and an incision[3] is made at the joint in the middle of the body; the body is then repeatedly bent back and forth to expel the blood. After the blood has stood for a short time a clot forms from which the clear blue serum may be poured off. To a portion of this sufficient ether is added to form a saturated solution; this is allowed to stand in a stoppered bottle with occasional gentle shaking, until the ether is dissolved. A number of glass tubes are prepared by sealing

---

[1] Alsberg, C. L., *J. Biol. Chem.*, 1915, xxiii, 495. Alsberg, C. L., and Clark, E. D., *ibid.*, 1910–11, viii, 1. Alsberg, C. L., and Clark, W. M., *ibid.*, 1914, xix, 503. Alsberg, *ibid.*, 77.

[2] Harvey, E. N., *J. Gen. Physiol.*, 1918, i, 133.

[3] This enters the heart cavity. It is desirable to cleanse and dry the surface around the joint before making the incision.

one end and fitting to the other a piece of rubber tubing which can be clamped shut. Various dilutions of the blood are made (with a saturated solution of ether in sea water), each of which is placed in a separate tube. In one tube undiluted blood is placed. All the tubes are clamped shut to prevent the evaporation of ether.

The ether prevents the decolorization of the blood and the various dilutions furnish shades of color which serve as standards in subsequent experimentation.

If fresh water organisms are used in the experiments the procedure must be modified. Since the salt content of the blood is approximately that of sea water it must be reduced by dilution or by dialysis. If this is carried beyond a certain point precipitation occurs and the color of the aerated blood is paler in consequence.

The organisms are placed in glass tubes, provided with rubber tubing as described above, and blood (diluted or undiluted) is added; the tubes are then clamped shut, taking care to exclude bubbles of air. The observer then notes the time required to produce a definite change[4] in color (as determined by comparison with the tubes containing blood to which ether has been added).[5] It is desirable to observe the tubes against a dark background, preferably while facing the source of light. The best results are usually obtained by viewing the tubes from the end. For this purpose the tubes are inverted so that the rubber tubing is below; the organisms sink into it and the rubber tubing may be temporarily pinched off above the organisms so they cannot be seen.

As soon as a definite change in color is observed the tubes containing the organisms may be opened and shaken with air so as to restore the original color. There must be a control tube containing blood without organisms or ether; if this control becomes decolorized the experiment must be rejected, unless the decolorization is so slow as not to interfere with the result. Freshly drawn blood does not decolorize except very slowly; this is also true of freshly dialyzed blood, and of blood heated[6] for 5 minutes to 60°C. Blood preserved

---

[4] The tubes should be shaken from time to time.

[5] A "Daylight" lamp was used in most of the experiments.

[6] Heating at lower temperatures (40–55°C.) for a longer time may be preferable.

by the addition of ether may be freed from ether by a current of air[7] and does not decolorize rapidly until it has stood for some time. Blood which has been carefully dried to a jelly-like mass may be kept in this condition; it dissolves readily on the addition of water. But if dried to a hard, brittle mass it does not readily dissolve.

Under favorable conditions blood containing certain organisms,[8] (such as bacteria, or young *Limulus* in the trilobite stage) is quickly decolorized (in some cases within 2 minutes) and the time required for decolorization is remarkably constant.[9]

When the normal rate of oxygen consumption has been established by repeated determinations, reagents may be added and changes in the rate may be determined. In this way the relative rate under the influence of the reagent may be ascertained without knowing the absolute amounts of oxygen consumed in either case.

A variety of other substances, such as indigo-carmine, methylene blue, malachite green, etc., are also reduced and it is possible that some of them may prove more useful than *Limulus* blood. Experiments on this subject are being continued.

SUMMARY.

The blood of the horseshoe crab (*Limulus*) absorbs oxygen and turns blue when shaken in air. In the presence of certain organisms which consume oxygen it is quickly decolorized. By measuring the time required for the change of color the rate of consumption of oxygen may be determined.

[7] The air should be filtered or washed to remove bacteria before entering the blood.

[8] The salt content must be adjusted to the needs of the organism; it may be quickly ascertained by titration.

[9] The normal rate is sometimes irregular, due to factors not fully understood. In these cases the results are rejected. The disturbing factors require further study.

COMPARATIVE STUDIES ON RESPIRATION.

I. INTRODUCTION.

By W. J. V. OSTERHOUT.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

In the course of studies on antagonism the writer made experiments on the action of antagonistic substances on respiration. As a result of his experiments he became dissatisfied with the existing means of studying respiration in plants and undertook to devise more satisfactory methods. At his suggestion Dr. Haas developed an indicator method[1] which proved to be very satisfactory for aquatic organisms. However, it had serious limitations which were subsequently removed by a method devised by the writer. The new method is accurate, rapid, and convenient, and can be used for organisms of all kinds.[2]

A series of studies has been made in the writer's laboratory by the use of these methods, the first of which are here brought together. The work of Mr. Gustafson deals with higher fungi, that of Mrs. Brooks with bacteria, that of Miss Thomas with flowering plants, and that of Miss Irwin with a variety of animal material. The work of Haas[3] on the marine alga *Laminaria* was carried out under the same conditions, and may therefore be compared with the results described in this series.

In the first investigations the technique described by Haas[1] was employed in most cases. Phenolsulfonephthalein was added to the liquid containing the organisms, and respiration was allowed to proceed until a definite change in acidity had occurred. Usually the organism was then placed in a fresh sample of the solution and the process was repeated until the normal rate of respiration was ascer-

[1] Haas, A. R., *Science*, 1916, xliv, 105.

[2] Osterhout, W. J. V., *J. Gen. Physiol.*, 1918, i, 17.

[3] Haas, *Science*, 1917, xlvi, 462; *Proc. Nat. Acad. Sc.*, 1917, iii, 688.

tained;[4] the reagent was then added and new measurements of respiration were made in the same manner as before.

The rate of respiration after the addition of the reagent is expressed in each case as per cent of the normal rate. The normal rate is usually determined by taking the average of successive determinations. The normal rate may be different in each experiment (on account of differences in the quantity or kind of material used), but it is in all cases taken as 100 per cent.

The relative rate was ascertained in all cases by comparing the times required to produce a definite change of acidity and not by a comparison of the changes in acidity produced in equal times. The former method compares the reaction velocities directly, while the latter may not.[5]

This method of comparison has the further advantage of making it unnecessary to know what change in pH value is produced by adding a definite amount of CO_2. For if we always start the measurement from the same pH value and carry it to the same endpoint, we can be certain that the same amount of CO_2 has been produced in each case, although we may not know what this amount is. It is, in fact, quite unnecessary to know it if we are comparing the times required to do equal amounts of work.

An additional advantage of this method is that an error in the buffer solutions does not affect the results, providing we use the same buffer solutions throughout. For this purpose they are made up in large quantities.[6] Two solutions are chosen, the pH value of one being taken as the starting point (to which the experimental solution is always brought when starting a measurement) and that of the other as the end-point (to which the experimental solution comes as the result of respiration).

Certain precautions which are essential to accurate work may be briefly mentioned, in addition to those described by Haas.[3] It is necessary to use the purest reagents and in particular to see that they are neutral (or practically so); stock solutions may be kept in Pyrex flasks or tubes to avoid alkalinity due to the glass. Slight departure

[4] Unless the normal rate was fairly constant the experiment was rejected.

[5] Osterhout, *Science*, 1918, xlviii, 172.

[6] Buffer solutions of phosphates or borates will keep for a long time.

from neutrality may be compensated by adding CO_2 to the tap water or removing CO_2 (by means of a current of air free from CO_2 or by a current of hydrogen). In this way the solution may be brought to the most convenient pH value; all experiments are then started at this value. It is desirable to choose a value in the region where the indicator is sensitive.

In case alkali is added to bring the solution to a given pH value it must be done before the normal respiration is measured, so that the buffer effect of the alkali will be the same in the measurements of normal and abnormal respiration.

The buffer effect of all reagents must be carefully measured. This may be done by means of an apparatus recently described.[7] In the case of the reagents employed in these studies there is practically no buffer effect (unless the contrary is expressly stated). The result of buffer action is to make the amount of respiration appear smaller than it really is.

It was found that no alkali or acid (other than carbonic) was given off by the organisms studied. This was determined by placing the organisms in tap water free from CO_2 and allowing them to respire for some time so as to change the color of the added indicator; the CO_2 was then driven off (by a current of air free from CO_2) and it was found that the indicator returned to its original color.

It was found by control experiments (in which the indicator was added after respiration took place) that the indicator itself (at the concentrations employed) had no effect upon respiration.

The accuracy of the measurements depends on developing skill in matching standard colors under uniform conditions. The standard colors are prepared by making a series of buffer mixtures[8] containing the same concentration of indicator as the experimental solutions and contained in Pyrex tubes of the same size as those in which the organisms are respiring. Various devices may be employed to make the matching more accurate. A background of white or light gray paper is used by many, while others prefer opal or ground glass. In some cases it is advisable to screen off the lower part of the tube or

[7] Osterhout, *J. Biol. Chem.*, 1918, xxxv, 237.

[8] Cf. Osterhout, W. J. V., and Haas, A. R. C., *Science*, 1918, xlvii, 420, footnote 7. Frequent renewal of the buffer solutions is advisable.

to place the tube in a hole bored in a block of wood and to view it through another hole bored at right angles to the first. The last method is very useful when there is a color (due to small suspended organisms or to the giving off of coloring matter by the organism) which is superimposed on that of the indicator. This color is contained in the tube which is put into the wooden block in line with the tube containing the buffer solution, so that the light passes through both tubes before reaching the eye. In this way the color of the indicator can be varied independently of the disturbing color, which is due to the organism.

Uniform conditions for comparison of colors were secured by the use of the "Daylight" lamp.[9] This gives a uniform source of light under which colors can be matched with sufficient accuracy, so that the investigator is free from disturbances due to fluctuations of daylight.

The best test of the accuracy of the work is to make repeated measurements on the same material in its normal environment at constant temperature. Tests made with favorable material show that the variation is very small, the probable error (in the time required to produce a standard change in acidity) being less than 1 per cent. Hence if greater variations are found, they must be due to the variability of the material, to the personal equation of the observer, or to unfavorable conditions (*e.g.* of illumination) in comparing colors, rather than to the method. A probable error of less than 1 per cent must be regarded as highly satisfactory for biological measurements.

The results obtained by the use of the new apparatus[2] are quite as accurate as those obtained by the original method.[1]

Attention may be called to two things in respect to the curves: (1) After each measurement there is an interval during which the solution is changed; since the organism is exposed to the action of the reagent during this interval the time should be included in the total time of exposure (as shown in Fig. 1). (2) The rate is obtained by taking the reciprocal of the time required to produce the standard

[9] Cf. Luckiesh, *Science*, 1915, xlii, 764. The form used is known as "north light."

change in acidity; hence it represents the average rate for that period. If the rate changes during the period, the average rate is probably the actual one somewhere near the middle of the period. Hence in Fig. 1, each ordinate representing the average rate is placed in the middle of the period. In these papers all curves in which rate is
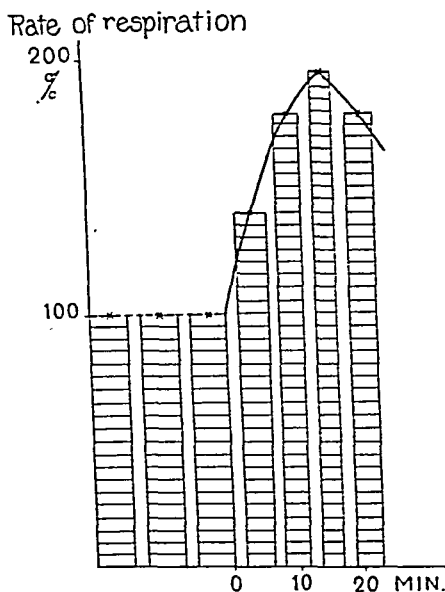
FIG. 1. Curve showing the rate of respiration under normal conditions (dotted line), and under the influence of a reagent (unbroken line). The periods during which measurements are made are indicated by horizontal shading; the intervals during which no measurements are made are left blank. Each measurement gives an average rate for the period; as this average rate is probably the actual rate near the middle of the period, the ordinate expressing the average rate is placed in the middle of the period. For convenience in comparison, time is reckoned from the beginning of exposure to the reagent.

plotted against time are made in this manner. For purposes of comparison the time expressed on the abscissæ is usually reckoned from the beginning of exposure to the anesthetic. The preceding part of the experiment, during which respiration was going on under normal conditions, is also represented on the curve but in this part the

figures expressing time are omitted from the abscissæ in order to avoid confusion.

In averaging curves the following procedure was adopted. A curve was drawn for each experiment (plotting rate of respiration as ordinates and time as abscissæ). These curves were averaged graphically, making use of interpolated points. It may be stated that this procedure is perfectly sound since the error of interpolation is usually less than the experimental error.

The rate of respiration is stated in all cases as per cent of the normal rate (which is always taken as 100 per cent). The rate is taken as the reciprocal of the time required to produce the standard change in acidity. The time required to produce this change under normal circumstances is usually stated, and from this the actual time required to produce the same change under the influence of the reagent may be easily calculated. It is not necessary to find the reciprocals of the time under normal and abnormal conditions and then to divide the latter by the former in order to get the relative rate; for the same thing is accomplished more quickly by dividing the time required to produce the standard change under normal circumstances by the time required to produce the same change under the influence of the reagent.

Of particular interest is the fact that these investigations were made by a method which enables us to ascertain the time curves of respiration by determinations made at very brief intervals (in some cases 1 minute or less). When the intervals are long (as in practically all previous investigations) it may happen that a rise in the rate of respiration which is quickly followed by a fall cannot be detected; and in general the form of the curve cannot be determined with sufficient accuracy to enable us to study the dynamics of the process.

Another point of importance is that so wide a range of material was studied that it is possible to judge whether the results obtained are of general validity. The plants included bacteria, the higher fungi, algæ, and flowering plants, while the animals included insects, frog eggs and tadpoles, and fish embryos. Precisely similar experiments were made on all these forms and as these experiments were all carried out under the same conditions the results are comparable in all respects.

All of these papers deal primarily with the effect of anesthetics on respiration. The special interest of this problem is too well known to require extensive discussion. Of late it has centered largely about the theory of Verworn, which states that anesthesia is a kind of asphyxia, due to a checking of respiration by the anesthetic.

Although this theory has been widely accepted there are excellent reasons for regarding it as invalid. Among these may be mentioned the observations of Warburg,[10] who found that phenylurethane inhibited cell division in the sea urchin egg without altering the consumption of oxygen, and those of Winterstein[11] who found that anaerobic animals are easily narcotized, which is difficult to understand if narcosis depends on interference with oxygen consumption. Loeb and Wasteneys[12] showed that to produce complete narcosis in *Fundulus* embryos by lowering the rate of respiration (by means of KCN) it was necessary to diminish respiration to one-fourteenth of the normal; but the same degree of narcosis could be produced by chloroform with a lowering of respiration amounting to only 5 per cent (or even less). In this case it would appear that anesthesia is not due to the checking of respiration by the anesthetic but to some other mode of action. This conclusion was confirmed by a variety of experiments made with other anesthetics and upon other organisms. Winterstein[13] in a later paper has shown that in the spinal cord of the frog, anesthetized with alcohol, the rate of respiration is above the normal.

The experiments upon plants made by various observers[14] are not in agreement. This is doubtless due to differences in the method of experimentation. One point of great importance which has been brought out in recent studies, particularly by those in this series,

[10] Warburg, O., *Z. physiol. Chem.*, 1910, lxvi, 305.

[11] Winterstein, H., *Biochem, Z.*, 1913, li, 143.

[12] Loeb, J., and Wasteneys, H., *J. Biol. Chem.*, 1913, xiv, 517; *Biochem. Z.*, 1913, lvi, 295.

[13] Winterstein, *Biochem. Z.*, 1914, lxi, 81.

[14] For a review of the literature see Czapek, F., Biochemie der Pflanzen, Jena, 2nd edition, 1913, i, 195 ff. See also Ewart, A. J., *Ann. Bot.*, 1898, xii, 415, and Appleman, C. O., *Am. J. Bot.*, 1916, iii, 223. For a general review of the literature on animals, see Winterstein, H., *Biochem. Z.*, 1913, li, 143; also Höber, H., Physikalische Chemie der Zelle und der Gewebe, Jena, 4th edition, 1914, 460 ff.

is that the result depends in most cases on the length of the experiment. This is due to the fact that under the influence of the anesthetic the rate of respiration constantly changes. In the older experiments this was overlooked and the discrepancies in the results are doubtless due in large measure to this circumstance. It is important to be able to determine the rate of respiration at intervals of 5 or 10 minutes, or even less, as is possible by the method employed in these studies.

All of the plants studied in the writer's laboratory (including representatives of bacteria, higher fungi, algæ, and flowering plants) agree in their behavior toward anesthetics. While lower concentrations produce no effect on respiration, higher concentrations cause a rise followed by a fall. In general the rise of respiration appears to be associated with reversible anesthesia while the fall below the normal rate indicates irreversible toxic effects, at least if it goes much below the normal.

It is evident that these results are directly opposed to the theory of Verworn.

The results obtained by Miss Irwin upon animals are likewise not in agreement with Verworn's doctrines, for although they show that anesthetics may produce a temporary decrease of respiration, which cannot be wholly explained on the ground of cessation of movement, this decrease is much too small to produce anesthesia.

On the other hand Miss Irwin's observations are interesting as showing an apparent difference between animals and plants in their behavior toward anesthetics, in that the temporary decrease followed by an increase which is found in animals does not occur in plants. If this should prove to be generally true, it is significant.

The facts developed in these studies are of considerable interest but it seems wiser to defer their interpretation until more information is available. They offer suggestions for further attacks upon the problem and have an important bearing on the theory of anesthesia. These investigations are being continued and it is expected that additional results will be forthcoming in the near future.

It may be added that throughout these articles the term respiration is used as meaning oxidative processes which furnish energy, beginning with the taking up of O_2 and ending with the production of

CO_2 and H_2O. The consumption of O_2 and the production of CO_2 furnish the most convenient means of measuring respiration and it is always desirable to study both together. If we find that O_2 is consumed but no CO_2 is produced, as in some of Warburg's experiments,[15] we may speak of respiration. If on the other hand CO_2 is produced but no free O_2 is consumed, as is the case in anaerobic respiration, we may regard this as respiration also. Ordinarily a rise in the rate of CO_2 output is interpreted as a rise in the rate of respiration, but it might happen that the sudden production of an acid (*e.g.* lactic acid) might set free CO_2 from carbonates already present in the tissue. When there is reason to suspect that this is the case special precautions must be taken to ascertain how the CO_2 is produced.

Some writers endeavor to make a distinction between oxidation in the living cell and that which occurs after death. In the opinion of the writer there is no valid ground for such a distinction, and the term respiration is here used to include processes which occur immediately after death.

SUMMARY.

A series of investigations on respiration with improved quantitative methods has been commenced. The first of these are here described. They show that when anesthetics are employed in sufficient concentration to produce any result, plants show a rise in the rate of respiration which is followed by a fall. In the animals studied, the rise (found in higher concentrations only) was preceded by a temporary fall which is not entirely due to lowering of muscular activity or tonus. In lower concentrations the effect on animals was merely a decrease of respiration.

The results of all the investigations are opposed to the theory of Verworn.

[15] Warburg, *Ergebn. Physiol.*, 1914, xiv, 319.
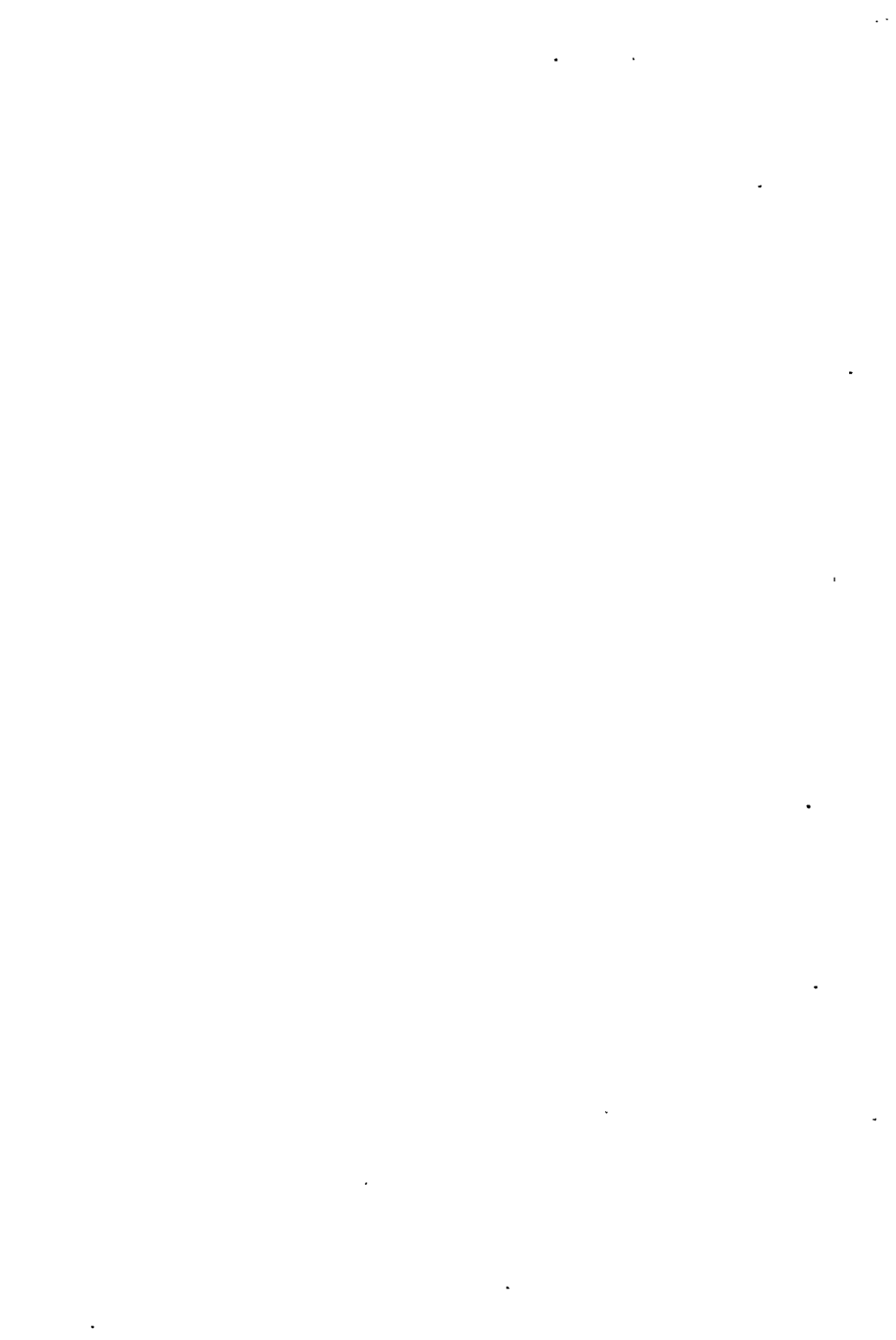
COMPARATIVE STUDIES ON RESPIRATION.

II. The Effect of Anesthetics and Other Substances on the Respiration of Aspergillus Niger.

By F. G. GUSTAFSON.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

The object of this investigation is to compare the action of anesthetics and some other substances on the respiration of a fungus with similar effects in other groups of organisms.

The fungus which has been used throughout these experiments is *Aspergillus niger*. This was selected because it grows well in a nutrient solution and forms on the surface of the liquid a compact mass of hyphæ, which is easily handled. *Penicillium* sp. was also tried, but this forms loose tufts of hyphæ, which are very hard to manipulate.

The fungus was grown in a nutrient solution of 40 gm. of cane sugar, 2 gm. of KNO_3, 1 gm. of KH_2PO_4, and 0.5 gm. of $MgSO_4$ in 800 cc. of tap water. This solution has an acid reaction, a fact which may have been beneficial in preventing the growth of bacteria. The cultures were examined at various times for bacteria but at no time were any found. The fungus was grown from spores in 100 cc. Erlenmeyer flasks, in about 30 cc. of sterilized nutrient solution. They were kept slightly above room temperature. Spores were usually formed in 3 or 4 days after inoculation, so it was found best to use the cultures when 2 days old. The respiration was at its maximum at this time. If older cultures were taken respiration was not so great.

The indicator method[1] was used for determining the amount of CO_2 given off. For buffer solutions borax and boric acid solutions were used in various mixtures. A table for making up buffers of

[1] Haas, A. R., *Science*, 1916, xliv, 105.

any desired pH value by means of borates and boric acid has been published by Palitzsch[2] and later republished by Osterhout and Haas.[3]

In the experiments 10 cc. of tap water were used in each tube to which five drops of a 0.01 per cent solution of phenolsulfonephthalein were added as indicator. That this indicator was not toxic to the fungus was proved by sowing spores in a nutrient solution containing the same concentration of indicator as used in the experiments. A control with the same amount of spores was inoculated at the same time and grown under similar conditions. The two cultures matured at the same time and behaved similarly when treated with anesthetic.

Respiration was measured in the nutrient solution and in sugar solutions in preliminary experiments, but as the results were the same as when tap water was used, the nutrient solution was replaced by tap water, which was easier to handle. The tap water was very near the neutral point and was brought to the desired alkalinity by boiling off some of the dissolved CO_2 or by washing out some of the CO_2 by means of a current of air free from CO_2.

When an experiment was started, some of the fungus was thoroughly rinsed in tap water to wash off the nutrient solution. Any adhering bubbles of gas enclosed in the interwoven hyphæ had to be got rid of before the readings were begun, either by gently squeezing the material between the fingers or by a continued shaking in several changes of water. The mass of hyphæ was then separated into pieces small enough to be shaken about easily in the tube. They were then put into a Pyrex glass tube which was closed at one end, while at the other was attached a paraffined rubber tube about 3 inches long.[1] Before putting the fungus in the tube, 10 cc. of tap water (plus five drops of indicator) had been poured in. When the fungus had been put in, the rubber tube was closed with a spring clamp, in such a way as to enclose a small bubble of air, to act as a stirrer. In all experiments this bubble was made as nearly the same size as possible. The tube was now shaken until any gas that may have adhered to the material was detached. The color of the experi-

[2] Palitzsch, S., *Biochem. Z.*, 1915, lxx, 333.

[3] Osterhout, W. J. V., and Haas, A. R. C., *Science*, 1918, xlvii, 420.

mental tube was then matched with that of a buffer tube of the same size (and having the same concentration of indicator), using a constant source of light ("Daylight" lamp).[4]

The alkalinity of the water was brought (by the means mentioned above) to a point a little above pH 7.60; after adding the material it was allowed to stand until it fell to pH 7.60, which was taken as the starting point in all experiments. The time was noted and the tube put aside for a minute or two, when it was gently shaken to distribute the CO_2 throughout the solution. The tube was again matched, this time with another buffer tube having a pH value of 7.25. If the experimental tube had not yet reached this, it was repeatedly examined at very short intervals until the two matched. The change in pH was from 7.60 to 7.25, which was the standard unit of measurement in all cases.

In starting an experiment the time required for this change was noted and the material was rejected unless this time was practically constant for at least three periods before any anesthetic was added. The time varied with the amount of material used, but was made as near to 3 minutes as possible in each experiment. When the endpoint was reached, the material was taken out and rinsed in tap water before starting a new measurement with a new solution.

When a practically constant rate of respiration had been obtained, the material was placed in a solution of the desired concentration of the anesthetic. After the addition of the anesthetic the respiration was measured in the same manner as before. The experiments with anesthetics lasted from half an hour to 2 hours.

On account of the short periods used it was found impossible to carry on a control simultaneously with the experiment. Therefore a large number of control experiments were made from a number of cultures and at different times during the period of investigation. These control experiments agreed very closely, so they were averaged to make up a control curve, which has been used in all figures. As will be noticed from the figures, the control shows a gradual decrease in rate of respiration. This is probably due to a decrease in oxidizable

[4] For other details see the introductory article by Osterhout, W. J. V., *J. Gen. Physiol.*, 1918, i, 171.

material within the cells, as this decrease did not occur when a 3 per cent sugar solution was used in place of tap water.

All the experiments were made at room temperature (18–20°C.).

In regard to the accuracy of the results it may be stated that in no case did the probable error exceed 2.4 per cent of the mean.

The measurement of respiration at frequent intervals, as here practised, has great advantages. Methods heretofore employed in measuring respiration have usually necessitated the use of long periods. It will be noticed readily from curves here presented that if periods of an hour's duration had been used no increase would have been found except in one or two cases, because the increase, though distinct enough, lasts but a short time (in many experiments only from 5 to 10 minutes). This increase is followed by a decrease, and if the total amount of CO_2 given off in 1 hour were measured, the large output for a short time would be more than counterbalanced by the longer period of small output, so that the total would be below normal, and we should record a decrease instead of an initial increase followed by a decrease. In the writer's opinion there is no doubt that this is what has happened in many cases, where only a falling off of respiration has been reported. Schroeder[5] got a decrease in respiration of *Aspergillus niger* when he used 6 and 7 per cent ether. In the present investigation 3.65 and 7.3 per cent ether both gave a decided increase (except in a few cases), but this was followed by a drop, and if 1 or 2 hour periods, as Schroeder used, had been employed, it is very likely that only a decrease would have been noticed. Kosinski[6] as the result of experiments with *Aspergillus niger* reports an increase in CO_2 output with 0.5 per cent ether and a decrease with 5 and 7 per cent; with 0.2 per cent cocaine and 0.02 per cent strychnine nitrate an increase was observed.

The first experiments were made with formaldehyde. A few experiments were conducted at first with 0.2 per cent (by volume), but as this concentration gave a very small change, 0.4 per cent was used. This stronger concentration gave a very large increase followed by an abrupt decrease. The results are shown in Fig. 1. Six experiments

[5] Schroeder, H., *Jahrb. wiss. Bot.*, 1907, xliv, 409.

[6] Kosinski, I., *Jahrb. wiss. Bot.*, 1902, xxxvii, 163.

were made with this concentration. Their average happens to correspond almost exactly with one experiment, and this experiment
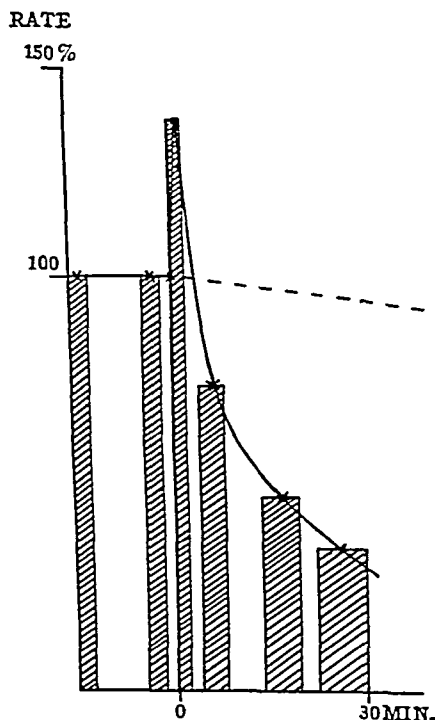
FIG. 1. Curve showing the effect of 0.4 per cent formaldehyde on the respiration of *Aspergillus niger*. The shaded portions represent the periods during which respiration was measured. The unshaded portions represent the intervening time during which no measurement was made (*e.g.*, time spent in changing the material from one solution to another). The horizontal part of the curve shows the normal respiration; the remainder the respiration in 0.4 per cent formaldehyde. The dotted line represents the respiration of a control in tap water. Time is reckoned from the beginning of the exposure to formaldehyde. The normal rate (which is taken as 100 per cent) corresponds to a change from pH 7.60 to pH 7.25 in 2.75 minutes.

was used instead of the average in drawing the curve, as will be seen in Fig. 1. The periods during which the measurements were made are indicated by shading. The rate which is obtained for each period

is an average for that period, and if the rate is changing, it is evident that this average rate will in general occur somewhere near the middle of the period.   Hence in drawing the curve the points are taken in the middle of each period.   The other curves are made in the same way but the periods are not indicated.

After exposure to the reagent has begun, the intervals between periods of measurement are included in the total time of exposure to the reagent, because even when the material is momentarily lifted out, it remains covered by a film of the reagent.   The relative rate of respiration for each period is obtained by dividing the rate for that period by the normal rate.   This applies to all the subsequent work.

The main part of the work was done with ether.   Various concentrations between 0.37 and 1.46 per cent (by volume) were tried in the · beginning but as these had little or no effect, higher concentrations were employed.   A slight increase of respiration, which lasted for some time, was obtained with 1.46 per cent ether.   With 3.65 per cent three distinct types of results were obtained.   The first and most common type was a sharp rise in respiration in the first period, followed by a gradual decrease (Fig. 2, Curve A), so that at the end of half an hour the rate was slightly below normal.   From this point on the rate decreased more slowly, reaching about 60 per cent of the initial rate at the end of 80 minutes.   A second type gave a curve with a flattened top having the maximum rate of respiration in the third or fourth period.   Only very few experiments showed this type. The third type never gave a rise, but always a slow decrease from the beginning (Fig. 2, Curve B).

These three types were constant in the sense that each culture always gave only one result, no matter how many experiments were made.   Though the cultures were grown under identical conditions, yet this difference in behavior was always noted.

A saturated solution of ether was also tried.   As this is approximately 7.3 per cent by volume, the latter designation has been used for the sake of convenience.   With 7.3 per cent only one type of result was noticed, even when the same cultures were used with which different results had been obtained with 3.65 per cent.   A very pronounced increase was noticed in the first period followed by an almost· equally

rapid decrease, so that in from 6 to 10 minutes the rate was back at normal. At the end of an hour respiration was nearly at a standstill (Fig. 2, Curve C).
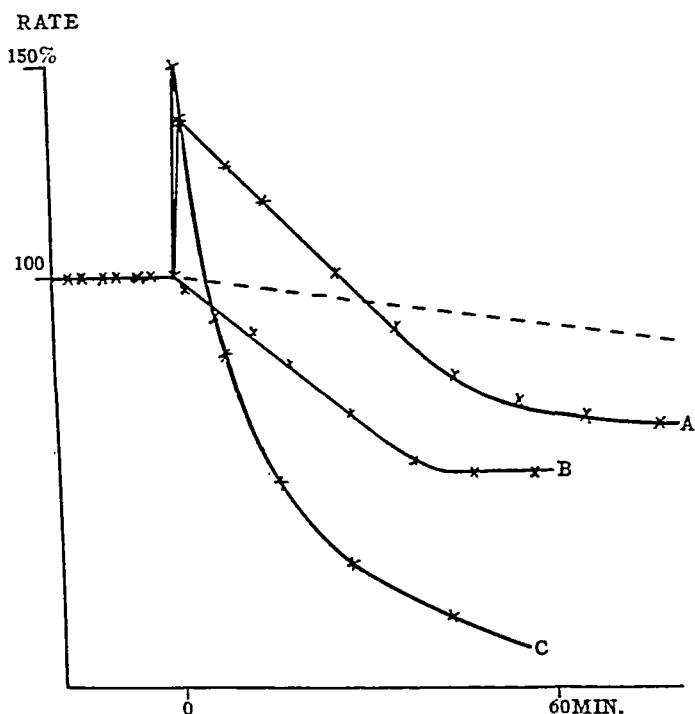
FIG. 2. Curves showing the effect of ether on the respiration of *Aspergillus niger*. Curve A, in 3.65 per cent ether; Curve B, a different culture in 3.65 per cent ether; Curve C, in 7.3 per cent ether. The horizontal part of the curves shows respiration in tap water before exposure to ether. Dotted line shows respiration of a control in tap water. The normal rate (which is taken as 100 per cent) corresponds to a change from pH 7.60 to pH.7.25 in 3.25 minutes for Curve A and in 3 minutes for Curves B and C. Curve A represents the average of four experiments; Curves B and C, the average of five experiments. Probable error, less than 2.4 per cent of the mean.

The results with ether are shown in a different manner in Fig. 3.

A number of experiments on recovery were conducted with 7.3 per cent ether. The result of these experiments was, that if respiration

had risen and fallen again to normal and the material was then taken out of the ether solution and put in a nutrient solution, it was found on observing it 2 to 3 hours later that respiration was practically normal (Fig. 4, Curve B).   If on the other hand the mater al was allowed to remain in the ether solution until the rate had fallen to about 60 per cent of the normal and then placed in nutrient solution recovery to normal was not obtained.   From this it would seem
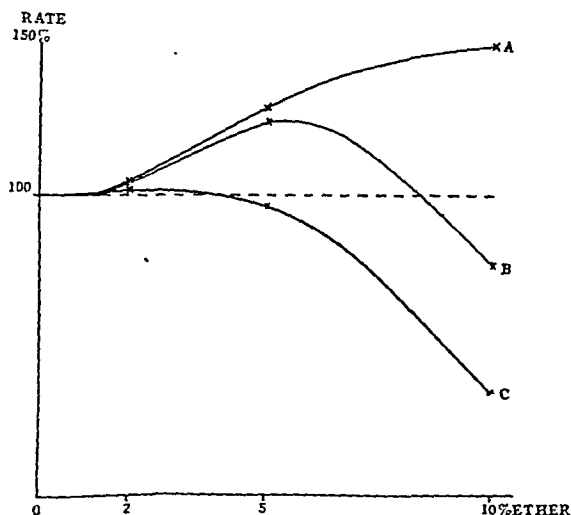
FIG. 3. Curve A shows the rate of respiration of *Aspergillus niger* in various concentrations of ether after an exposure of 2 minutes.   Curve B shows rate of respiration after an exposure of 10 minutes.   Curve C shows rate of respiration after an exposure of 30 minutes.   The dotted line shows the normal respiration.

The figures expressing the per cent of ether should be corrected by multiplying by 0.73.

that only the increase in respiration is reversible and that when a decrease takes place an irreversible reaction involving injury is going on.

A 20 per cent solution (by volume) of acetone was also employed. A very large increase took place, with the maximum during the second period (Fig. 5, Curve B).   This concentration of acetone was very much less toxic than 7.3 per cent ether.

Alkaloids usually have a special effect.   For this reason it was thought best to try caffeine as a representative of this group.   Low

concentrations, such as Haas[7] reports using, had no effect whatsoever. The lowest concentration that showed any effect was 0.5 per cent (Fig. 5, Curve A). This gave a decrease in respiration from the beginning. In the first two periods there was a decrease of nearly 20 per cent below normal; then followed several periods with hardly any decrease at all. Several experiments with a saturated solution
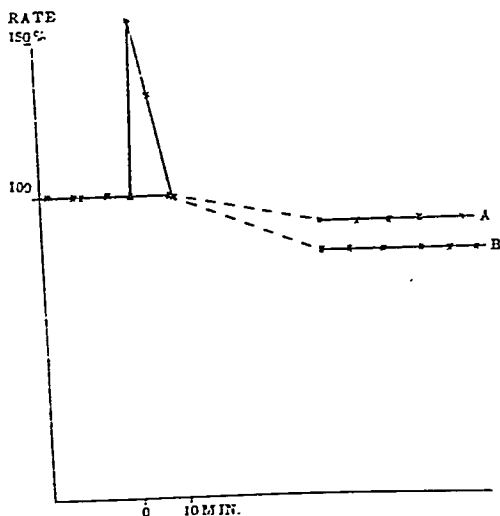
FIG. 4. Curve B shows the respiration of *Aspergillus niger*, first for 20 minutes in tap water (horizontal unbroken line), then for 9.5 minutes in 7.3 per cent ether, then for 130 minutes (interval shortened in figure to save space and denoted by dotted lines) in nutrient solution, and finally for 36 minutes in tap water (unbroken line). Curve A shows the respiration of a control placed in tap water for 30 minutes, then for 130 minutes in nutrient solution (dotted line), then for 34 minutes in tap water (unbroken line). The normal rate (which is taken as 100 per cent) corresponds to a change from pH 7.60 to pH 7.25 in 3 minutes. Average of three experiments. Probable error less than 2.4 per cent of the mean.

of caffeine were also performed. An initial increase amounting to about 15 per cent above normal was noticed (Fig. 5, Curve C). This was followed by a gradual decrease till a rate of 60 per cent of the normal was reached, when the rate of respiration became stationary and remained so till the end of the experiment (over half an hour).

[7] Haas, *Science.* 1917. xlvi, 462.·
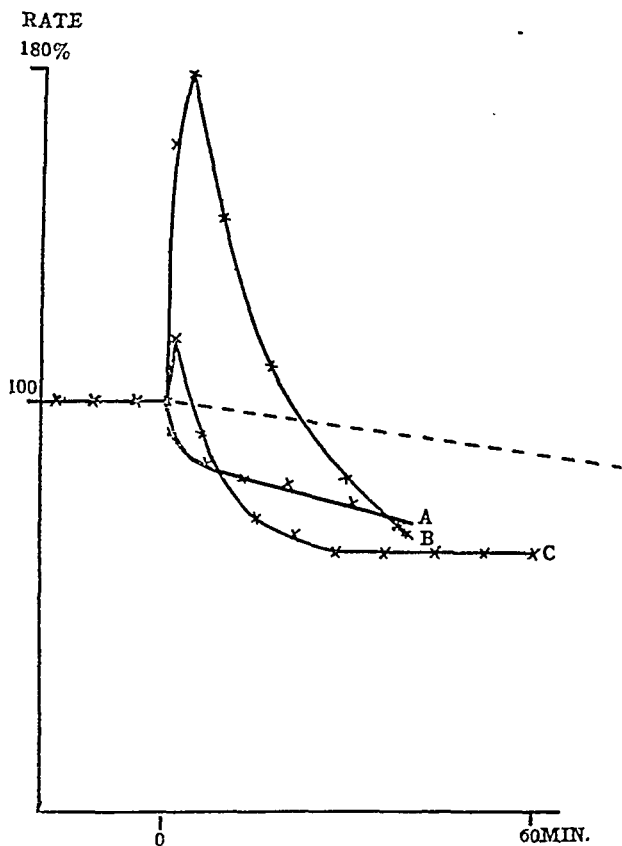
FIG. 5. Curve B shows the respiration of *Aspergillus niger*, first for 20 minutes in tap water (horizontal part of curve), then in 20 per cent acetone. Curve A shows the respiration, first in tap water (horizontal part of curve), then in 0.5 per cent solution of caffeine. Curve C shows respiration, first in tap water (horizontal part of curve), then in saturated solution of caffeine. The dotted line shows the respiration of a control in tap water.

The normal rate (which is taken as 100 per cent) corresponds to a change from pH 7.60 to pH 7.25 in 2.75 minutes for Curve A; in 3.25 minutes for Curve B, and in 3 minutes for Curve C.

Curve A represents the average of seven, Curve B of four, and Curve C of two experiments. Probable error less than 2.4 per cent of the mean..

The action of caffeine seems to be very much slower and less pronounced than that of the other substances tried.

The absence of any noticeable buffer action in any of the solutions was determined by means of an apparatus which has been described by Osterhout.[8] A measured amount of CO_2 is introduced into a certain amount of solution with indicator and the change in pH is noted. This is compared with the change caused by an equal amount of CO_2 in the same amount of tap water.

The above results agree in the main with those obtained by Haas[7] on *Laminaria*. Usually stronger solutions were needed to cause any effect on the respiration of *Aspergillus niger*, and the changes were not so great. With all reagents tried there was an increase, though with some concentrations only a decrease was noted.

SUMMARY.

1. In concentrations which are high enough to produce any effect, formaldehyde, ether, and acetone cause an increase, followed by a decrease, in the rate of respiration.

2. 3.65 per cent ether, which causes an increase with certain cultures, produces only a decrease with others.

3. The reaction producing an increase in the respiration with 7.3 per cent ether is a reversible process, while the reaction producing the decrease is not reversible.

4. 0.5 per cent caffeine produces only a decrease in respiration while a saturated solution causes an increase, which is followed by a decrease.

[8] Osterhout, *J. Biol. Chem.*, 1918, xxxv, 237.

COMPARATIVE STUDIES ON RESPIRATION.

III. The Effect of Ether on the Respiration and Growth of Bacillus Subtilis.

By MATILDA MOLDENHAUER BROOKS.

(*From the Laboratory of Plant Physiology, Harvard University, Cambridge.*)

Although so much stress is laid on the physiological reactions of bacteria in their identification and classification, little investigation (except to distinguish aerobic from anaerobic forms) has been devoted to the fundamental process of respiration in these organisms. In order to throw some light on the nature of this process and to compare the results with those obtained with other plants and with animals, a series of studies upon the respiration of bacteria has been undertaken. The present paper describes the results of some experiments on the effects of ether.

The organism selected for experimentation was *Bacillus subtilis*, isolated from water and inoculated upon agar-agar daily during the month previous to experimentation, so that a pedigreed culture was at hand. Several other organisms were tried; namely, *Bacillus mycoides, Staphylococcus pyogenes albus, Staphylococcus citreus, Bacillus typhosus;* but *Bacillus subtilis* was preferred because it was easier to handle. Bacteria were incubated for 16 to 18 hours at 37°C. and were then washed into a test-tube with a few cc. of tap water containing indicator. A thick emulsion was made by repeatedly drawing the liquid into, and ejecting it from a pipette which was drawn out to a very fine point.

The respiration of the bacteria was tested in boiled tap water. The boiling rendered the water alkaline by driving off the CO_2. To each 2 cc. of solution was added one drop of a 0.01 per cent aqueous solution of phenolsulfonephthalein. The color of the solution was compared with the colors of a set of standard buffer solutions, made according to Sörensen's directions with KH_2PO_4 and Na_2HPO_4, hav-

193

ing the same concentration of phenolsulfonephthalein as the experimental solution and contained in Pyrex tubes of the same size. The tap water was found to have a pH value of 8.3.

All experiments were done in Pyrex tubes each containing a total of 5 cc. of liquid as follows: Various amounts of tap water plus three standard drops of bacterial emulsion and sufficient saturated solution of ether in tap water to make the following concentrations of ether by volume: 0.037, 0.183, 0.329, 0.438, 0.95, 1.46, 2.9, 4.38, 5.84, and 7.3 per cent.

Each tube, therefore, contained a total volume of liquid amounting to 5 cc. in which the concentration of indicator was the same. The number of bacteria was made as nearly equal as possible in the different tubes by taking a uniform emulsion, mixing it thoroughly before taking the drops from it, and using a standard dropper so as to have the drops of equal size. The emulsion was transferred almost simultaneously to all the tubes. The tubes were then quickly closed (by clean corks boiled in paraffin) with the exclusion of all air and determinations were made by comparison with the standard set of buffer solutions contained in tubes of the same size. In every case there was a control consisting of organisms in tap water without ether, and of ether in tap water without organisms.

To see if phenolsulfonephthalein is toxic to bacteria, several trials were made by placing bacteria in a liquid of known pH value and after respiration had gone on for a definite time adding the indicator, and comparing that result with the color of a control tube to which the indicator was added at the beginning of the experiment. No difference in rate of respiration was observed.[1]

It was also found that ether has no buffer action to interfere with the measurement of the rate of respiration.

That the bacteria under these conditions give off no alkali or acid (other than carbonic) is shown by driving off the CO_2 at the end of the experiment. The solution then returns to the original pH value.

A great deal of variation in cultures of different ages was observed. Those more than 24 hours old have a markedly lower rate

---

[1] For other details see Paper I of this series (Osterhout, W. J. V., *J. Gen. Physiol.*, 1918, i, 171.

of respiration than cultures between 18 and 24 hours old. The time required to change the pH value from 8.3 to 8.1 by respiration may be only a few minutes when the culture is young, while as much as 6 hours may be necessary in the case of an older culture. This might be due to the fact that in the older culture the bacteria have gone into the resting stage or have produced spores. In this stage ether has little or no action upon the respiration of the bacteria.

The temperature maintained was from 18–20°C. as this was found to be sufficiently constant. Presumably the respiration of bacteria has the ordinary temperature coefficient of 2. A few experiments were tried at about 1°C. and it was found that exposure to this temperature for 15 minutes had a marked after effect, as little or no respiration was observed for about 6 hours.

In the earlier experiments it was thought desirable to permit respiration to produce a definite change in pH value and then wash out the CO_2 with H_2 or with air free from CO_2. But this was found to injure the bacteria (owing probably to mechanical or chemical disturbances), so that the rate of respiration was greatly diminished. Therefore it was necessary to use separate tubes for the control and for each concentration of ether (instead of finding the normal rate of respiration of a given tube and afterward exposing it to ether). It was possible to do this as a number of readings showed a fairly constant rate of respiration.

The rate is obtained by taking the reciprocal of the time required to produce a given change in pH value. It is expressed as per cent of the normal rate which is always taken as 100 per cent.

Fig. 1 shows a comparison between the respiration of *Bacillus subtilis* in tap water and in four concentrations of ether (by volume). In every case the rate is more rapid at first and becomes slower as the reaction of the medium becomes more acid. The normal curve, indicated by the dotted line, shows a slower rate than any of the other curves. In no case does the indicator show the pH value to be below 6.8 to 6.4.

When higher concentrations are used, the respiration is more rapid during the first few pH intervals and much slower during the last few. In fact, concentrations of ether as high as 1.46 per cent and upward never reach so low a pH value as concentrations below 1.46 per

cent.   For example, the curve readily shows that 7.3 per cent ether,. although causing a rapid respiration at first, does not produce a lower pH value than 7.9.   After allowing these tubes to stand several days, they change about 0.2 pH unit, or reach 7.7 pH, owing probably to the fact that there are a few surviving organisms that are perhaps more resistant than the majority, as later experiments indicate.
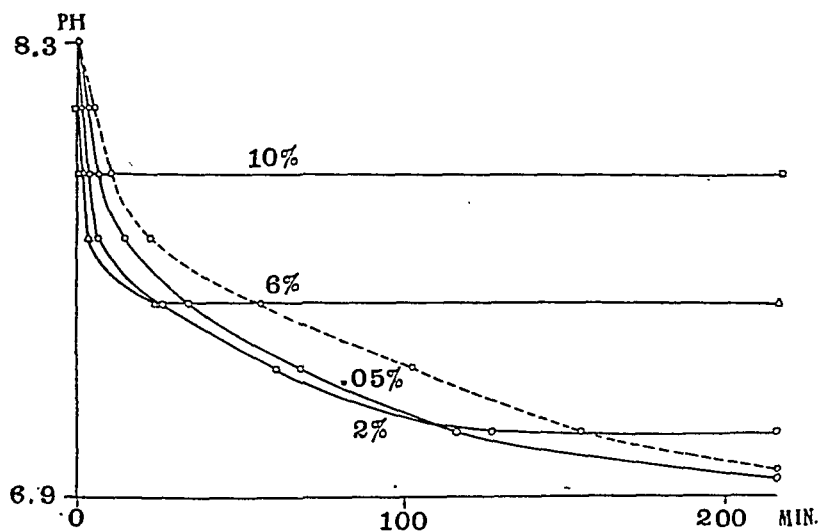
FIG. 1. Curves showing the effects of various concentrations of ether on the respiration of *B. subtilis*.   Dotted line, control.   Average of two experiments.
The figures expressing the per cent of ether should be corrected by multiplying by 0.73.

Fig. 2 shows more readily the effect of 7.3 per cent ether upon the rate of respiration as compared with the normal rate (dotted line). There is a sudden outpouring of CO_2 followed by cessation of respiration.

Fig. 3 plotted in the same way, shows the effect of lower concentrations of ether upon the rate of respiration as compared with the normal curve (dotted line).

Curve 1 in Fig. 4 shows the rate of respiration in various concentrations of ether during the first interval in which the pH value changes

from 8.3 to 8.1. The rate is taken as the reciprocal of the time required to change the pH value from 8.3 to 8.1. The curve shows a gradual increase in respiration as the concentration of ether increases. The curve rises more rapidly from 2.9 to 5.84 per cent, and very rapidly near 7.3 per cent. In fact, the change in pH value at 7.3 per cent is almost instantaneous.
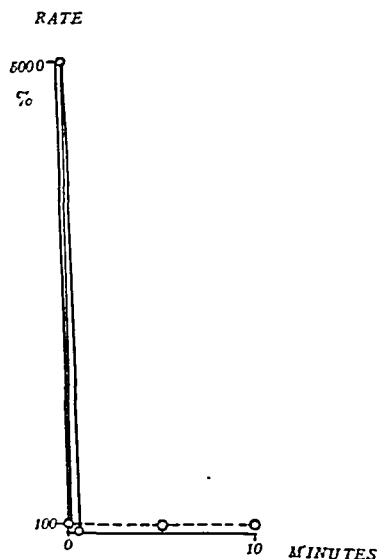
Fig. 2. Curves showing the effect of 7.3 per cent ether on the rate of respiration of *B. subtilis*. Dotted line, control. The normal rate (which is taken as 100 per cent) corresponds to a change from pH 8.3 to pH 8.1 in 7 minutes. Average of three experiments.

For the sake of comparison, a similar series of observations was made with the same concentrations of ether dissolved in 0.85 per cent NaCl. Curve 2 of Fig. 4 shows increase in respiration with increase in concentration; the curve rises rapidly at first, then more slowly and finally quite steeply. No sudden outpouring of CO_2 is observed in 7.3 per cent ether as is the case in the tap water (Curve 1). This seems to indicate an antagonism between the action of NaCl and ether.

The question arises whether the sudden outpouring of CO_2 in 7.3 per cent ether is due to the sudden production of a great excess of CO_2 or merely to the sudden liberation of CO_2 previously stored up in the cells (either as CO_2 or in the form of carbonates and bicarbon-
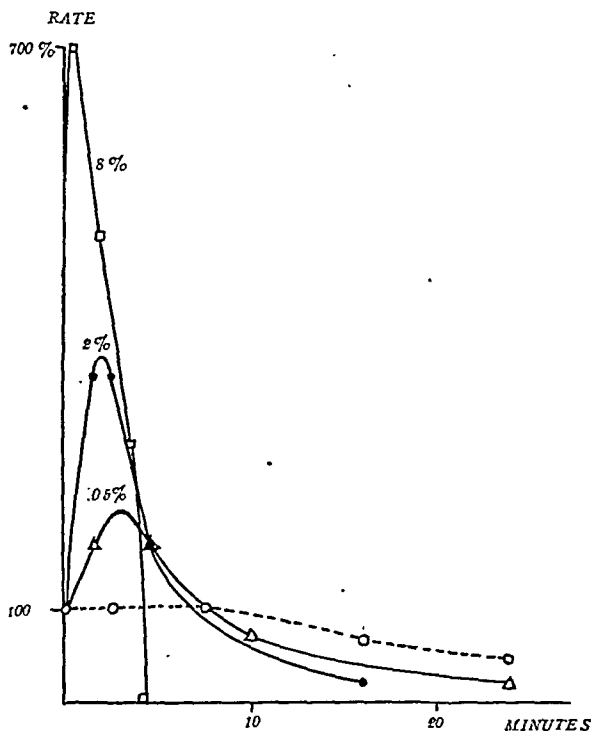
FIG. 3. Curves showing the effect of ether on the rate of respiration of *B. subtilis*. Dotted line, control. The normal rate (which is taken as 100 per cent) corresponds to a change from pH 8.3 to pH 8.1 in 5 minutes. Average of two experiments.

The figures expressing the per cent of ether should be corrected by multiplying by 0.73.

ates). It seems more probable that the latter is the case but there is also a sudden increase in O_2 consumption when the bacteria are placed in 7.3 per cent ether, as is shown by using *Limulus* blood as an indicator.[2]

[2] Osterhout, *J. Gen. Physiol.*, 1918, i, 167.

The great increase in respiration in 7.3 per cent ether in tap water raises the question whether the bacteria were injured. To obtain some light on this question, the contents of each tube were tested (after an exposure to ether lasting 20 minutes) by plating the bacteria on Petri plates and counting the colonies. Two loopfuls of solution from each Pyrex tube were diluted in 10 cc. of sterile water and from
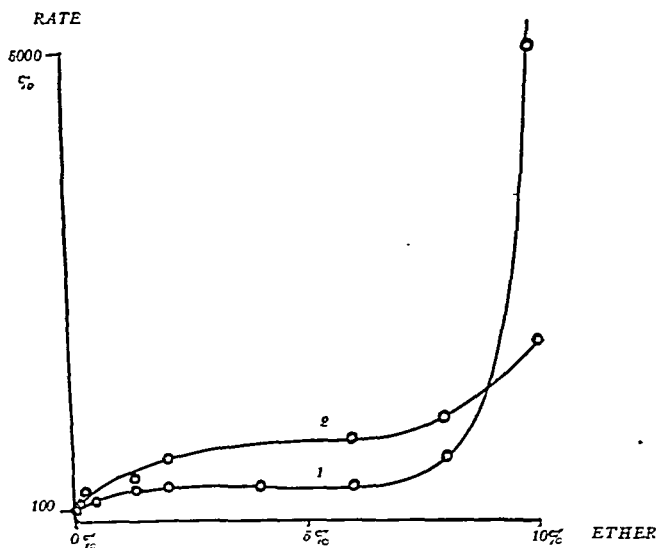
FIG. 4. Curves showing the effect of various concentrations of ether on the rate of respiration of *B. sublilis* in tap water (Curve 1) and in 0.85 per cent NaCl (Curve 2). The normal rate (which is taken as 100 per cent) corresponds to a change from pH 8.3 to 8.1 in 5 minutes. Average of three experiments.

The figures expressing the per cent of ether should be corrected by multiplying by 0.73.

these, one loopful was placed in 10 cc. of agar-agar and plates were poured in triplicate. These plates were incubated over night at 37°C. and counted the next day. The results were expressed as per cents of the normal. These per cents are plotted as ordinates in Fig. 5 and the concentrations of ether as abscissæ. In performing this experiment all precautions were observed to ensure sterility of the media.

To show what effect a longer exposure to ether would have upon the bacteria, another set of plates was poured in the same manner as the first set except that the bacteria were allowed to remain in ether for 1 hour.   The solutions tried were (1) tap water, (2) saturated solution of ether (about 7.3 per cent in tap water), (3) 0.85 per cent
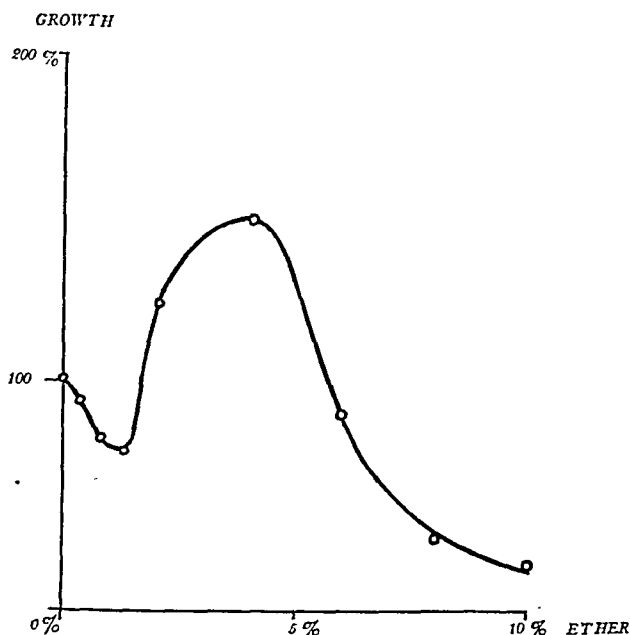
FIG. 5. Curve showing the effect of various concentrations of ether on the growth of *B. subtilis*.   The ordinates denote the number of colonies expressed as per cent of the normal, which is taken as 100 per cent.

The figures expressing the per cent of ether should be corrected by multiplying by 0.73.

NaCl in tap water, and (4) saturated solution of ether in 0.85 per cent NaCl solution.   The following results were obtained, averaging nine trials and counting tap water as 100 per cent.

Tap water.	Ether in tap water.	0.85 per cent NaCl.	Ether in 0.85 per cent NaCl.
per cent	*per cent*	*per cent*	*per cent*
100	10	112	14

Fig. 5 shows that as the concentration of ether increases up to 1.1 per cent there is a decrease in rate of growth, reaching a minimum at about 0.95 per cent. Concentrations of ether from 1.1 to 4 per cent produce increase in growth with a maximum at 2.9 per cent. All higher concentrations of ether produce decrease in growth. These observations show that ether is toxic in low concentrations and in very high concentrations, while intermediate concentrations stimulate growth.

A possible correlation between Fig. 4 and Fig. 5 may be made. In Fig. 4 there is a steeper ascent in the curve up to 1.1 per cent concentration of ether, and this corresponds to the first period of toxicity in Fig. 5. Then there follows a more uniform increase in respiration in Fig. 4 corresponding to an increase in growth in Fig. 5. Above 2.9 per cent ether, Fig. 4 shows the very steep ascent of the curve (or very rapid respiration) and Fig. 5 shows a sudden descent of the curve with a decrease in growth or death of the bacteria.

SUMMARY.

1. In all the concentrations of ether studied (from 0.037 to 7.3 per cent) there is an increase in the rate of respiration of *Bacillus subtilis* followed by a decrease.

2. In 7.3 per cent ether in tap water there is an extraordinary increase in the output of CO_2 (amounting to 50 times the normal). This does not occur when 0.85 per cent NaCl is added, which indicates antagonism between ether and NaCl.

3. Ether is toxic in low concentrations (0.037 to 1.1 per cent) and high concentrations (3.65 to 7.3 per cent) but in intermediate concentrations (1.1 to 3.65 per cent) stimulates growth.

COMPARATIVE STUDIES ON RESPIRATION.

IV. THE EFFECT OF ETHER ON THE RESPIRATION OF WHEAT.

BY HELEN STILLWELL THOMAS.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

Investigations on the effect of anesthetics on the production of CO_2 in higher plants have yielded somewhat contradictory results. Appleman[1] found that the respiration of potatoes exposed to ethyl bromide vapor for half an hour was approximately doubled. Irving[2] noted that the respiration of barley shoots was first increased and then decreased by chloroform vapor. Laurén[3] stated that some seedlings showed an increase in respiration when exposed to ether or chloroform, provided the treatment was not carried far enough to injure the plant permanently; other seedlings showed a decrease in respiration. Elfving[4] also observed an increase in respiration with ether and chloroform. Ewart[5] found that chloroform increased the respiratory activity in *Elodea*.

On the other hand, no increase in respiration was observed by Detmer,[6] nor by Bonnier and Mangin.[7] Tashiro[8] states that the respiration of dry seeds under the influence of anesthetics was decreased.

In no case where only a decrease was found was the time curve determined, and it is therefore impossible to say whether there was a preliminary increase. In Irving's experiments, where time curves

---

[1] Appleman, C. O., *Am. J. Bot.*, 1916, iii, 223.

[2] Irving, A. A., *Ann. Bot.*, 1911, xxv, 1077.

[3] Laurén, W., Über den Einfluss von Ätherdämpfen auf die Atmung von Keimlingen, Dissertation, Helsingfors, 1891 (*Bot. Jahresber.*, 1892, xx, 92).

[4] Elfving, *Oefversigt Finska Vetensk. Soc. Forh.*, 1886, xxviii.

[5] Ewart, A. J., *Ann. Bot.*, 1898, xii, 415.

[6] Detmer, W., *Landw. Jahrb.*, 1882, xi, 227.

[7] Bonnier, G., and Mangin, L., *Ann. sc. nat.*, 1886, cxi, series 7, 16.

[8] Tashiro, S., *Am. J. Physiol.*, 1913–14, xxxii, 107.

were determined, the periods were usually comparatively long, 1 or 2 hours, and therefore the complete series of changes in the rate of respiration could not be followed.

The material chosen for these experiments was wheat, as representing the flowering plants. The anesthetic used was ether in 7.3 and 3.65 per cent solutions, by volume.

Wheat seeds were soaked for 12 hours, during which time the process of germination enters upon its first stages. Seeds were selected which were as nearly alike as possible; twenty-five were placed in a Pyrex tube with 10 cc. of water containing five drops of the indicator, 0.01 per cent phenolsulfonephthalein. The tube was closed so that all air was excluded and the seeds themselves acted as a stirrer.

It·was found convenient to allow the seeds to give off enough CO_2 to change the solution from a pH value of 7.78 to a pH value of 7.36. This range of color change was arbitrarily selected as definite and easy to read, and formed the standard for all the experiments. Tap water which had been boiled to drive off the CO_2 sufficiently to give a pH value slightly higher than 7.78 was used in all cases. At the end of each period the liquid was poured out and fresh tap water was added. A normal rate of respiration was first established by repeated determinations,[9] then the reagent was added and the rate again determined. All results were expressed in per cent of the normal rate, which was called 100 per cent.

In these experiments the colors were matched with the aid of a "Daylight" lamp, which gave a constant source of light. The experiments were made at room temperature, about 18–20°C.[10]

The stock solution of ether was a saturated solution in tap water (containing the indicator of the standard concentration). In order to have the solution of the right pH value at the start (pH 7.78), two bottles of 7.3 per cent ether were kept on hand;[11] these had different pH values and by mixing, the desired value was attained.

[9] This was taken as the reciprocal of the time required to change the pH value from 7.78 to 7.36.

[10] For other details of technique see Paper I (Osterhout, W. J. V., *J. Gen. Physiol.*, 1918, i, 171.

[11] Glass stoppered bottles of Kavalier glass (which does not give off sufficient alkali to interfere with the experiment) were employed for this purpose.

In 7.3 per cent ether solutions there was an immediate increase in the rate of respiration which reached a maximum of 206 per cent (106 per cent increase) in 8 to 9 minutes (Fig. 1, Curve A). This
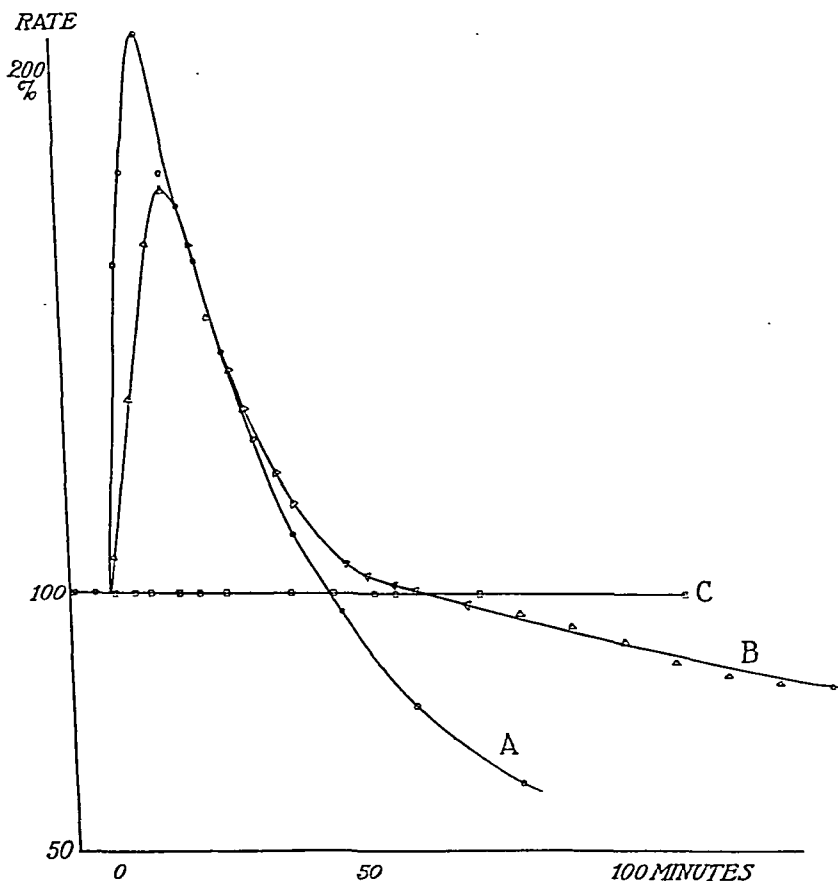
FIG. 1. Curves showing the rate of respiration of wheat seeds in 7.3 per cent ether solution (Curve A); in 3.65 per cent ether (Curve B); and in tap water (Curve C). Time is reckoned from the beginning of exposure to the ether solutions. The normal rate (which is taken as 100 per cent) corresponds to a change from pH 7.78 to pH 7.36 in 1.93 minutes for Curve A, in 2.28 minutes for Curve B, and in 1.53 minutes for Curve C. Curve A represents the average of ten, Curve B of five, and Curve C of four experiments. Probable error is less than 5 per cent of the mean.

was followed by a more gradual fall which reached the normal rate in 43 minutes and continued to fall, so that at the end of 90 minutes the rate was only 60 per cent of the normal.

Some experiments were made to determine the recovery after exposure to 7.3 per cent ether for periods of various lengths. This was done by putting the seeds in large Petri dishes to germinate. It was found that the seeds would not recover if the period of exposure was greater than 30 minutes. Short exposures (up to 8 minutes) were stimulating, for growth was more rapid than the normal growth of unexposed seeds, but any exposures beyond 8 minutes resulted in a decreased rate of growth up to 30 minutes, after which there was no recovery. During the period from 30 minutes to 43 minutes, the curve shows that respiration is above normal, but the seeds would not recover. This was probably due in part to rapid respiration after death, such as Haas[12] found in *Laminaria*, but it should not be inferred that death took place at the end of 35 minutes because no recovery was possible after this period. It is more probable that the seeds were alive at the end of 35 minutes and that death occurred after removal from the ether solution.

In the experiments in which 3.65 per cent ether solutions were used there was an immediate increase which reached the maximum of 178 per cent (78 per cent increase) in 13 minutes (Fig. 1, Curve B). This increase was followed by a more gradual fall which reached the normal rate in 1 hour, and then fell still more gradually below normal so that at the end of 140 minutes the respiration was only 81 per cent of the normal. In all experiments with 3.65 per cent ether the seeds showed by the germination test that the anesthetic did not cause death, even after an exposure of 12 hours.

The 3.65 per cent ether caused a more gradual rise and fall in the rate of respiration than the 7.3 per cent ether and the increase was not so great. In both cases, however, there was a definite rise followed by a fall.

In 0.73 per cent ether the rise was less than in 3.65 per cent and recovery was possible even after an exposure of 12 hours.

[12] Haas, A. R. C., *Proc. Nat. Acad. Sc.*, 1917, iii, 688.

The consumption of oxygen was determined by Winkler's method.[13] During an exposure of 30 minutes to 7.3 per cent ether the consumption of oxygen was 145 per cent of the normal (average of four experiments) while the production of CO_2 was 165 per cent of the normal.

SUMMARY.

These experiments show that 7.3 and 3.65 per cent ether solutions cause an increase in respiration followed by a decrease.   The results agree with those of Haas[14] on *Laminaria*, of Gustafson[15] on higher fungi, and of Mrs. Brooks[16] on bacteria.

They do not agree with the theory of Verworn that anesthesia is a kind of asphyxia and that it decreases respiration.

[13] Cf. Osterhout, W. J. V., and Haas, A. R. C., *J. Biol. Chem.*, 1917, xxxii. 141.
[14] Haas, *Science*, 1917, xlvi, 462.
[15] Gustafson, F. G., *J. Gen. Physiol.*, 1918, i, 181.
[16] Brooks, M. M., *J. Gen. Physiol.*, 1918, i, 193.

COMPARATIVE STUDIES ON RESPIRATION.

V. The Effect of Ether on the Production of Carbon Dioxide by Animals.

By MARIAN IRWIN.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

The purpose of this investigation is to make a comparison of the CO_2 output of animals with that of plants under the influence of a typical anesthetic such as ether. One obstacle to such a comparative study is found in the fact that the movements of animals are more or less inhibited by anesthetics; such inhibition diminishes the CO_2 output even when it is not due to anesthetics. Hence it is difficult to say how the CO_2 output would be affected by anesthetics if this complication were absent. This difficulty is largely avoided in my studies on frog eggs and *Fundulus* embryos, while in the investigations on tadpoles and on aquatic insects it is partly obviated by the method of experimentation.

No attempt has been made to compare the results with those obtained on animals by other workers, such as those of Tashiro,[1] because the methods were so different as to render a comparison difficult.

Method.

The method used in determining the output of CO_2 is the one described by Haas.[2] The standard buffer solutions were made with mixtures of phosphates. To 10 cc. of the solution, three drops of 0.01 per cent solution of phenolsulfonephthalein were added; in all the experiments sufficient NaOH was added to bring the pH value to 8.0. As the concentration of NaOH was the same in all cases the

---

[1] Tashiro, S., A chemical sign of life, Chicago, 1917.

[2] Haas, A. R. C., *Science*, 1916, xliv, 105.

buffer action was identical in the solutions containing anesthetics and in the controls (the ether has practically no buffer action).

In each case 10 cc. of the solution were placed in a Pyrex glass tube of the same diameter and thickness as the Pyrex glass tubes containing the buffer solutions. The organisms were put in the solutions and the time required to change from pH 8.0 to 7.7 was recorded by means of a stop-watch. After experimentation the solutions were tested by driving out the CO_2 by means of air free from CO_2; invariably the solutions returned to pH 8.0, thus proving that no acids other than the carbonic were responsible for the change in the pH value.

The temperature was kept approximately constant at 20°C. (with variations of less than 1°C.) by means of a water bath.

In presenting the results the rate of respiration is taken as the reciprocal of the time required to change the solution from pH 8.0 to 7.7. For convenience this rate is expressed as per cent of the normal rate, which is always taken as 100 per cent. The normal rate was in most cases practically constant.[3]

Frog Tadpoles.

The first set of experiments to determine the effects of ether on the carbon dioxide output of frog tadpoles was made with ten concentrations of ether, ranging from 0.007 to 0.55 per cent by volume. The tadpoles chosen were about 20 mm. in length; at this stage, they respire through the internal gills and the skin. A single tadpole was placed in each tube containing 5 cc. of liquid, and the time required to change the solution from pH 8.0 to 7.7 was noted. Fig. 1 (Curve B) shows the effect of ether on respiration, after an exposure of 150 seconds; there was no acceleration of respiration at any concentration: on the contrary there was a decrease from 0.15 per cent ether and upward. It might be thought that this was due to the cessation of the breathing movements, by which the water is carried to the gills, or to the stopping of the muscular movements, especially of the tail. As a matter of fact, however, at these concentrations and

[3] For further details see Paper I (Osterhout, W. J. V., J. Gen. Physiol., 1918, i. 171).

with this length of exposure these movements remained practically normal. The measurements of the breathing movements were made by determining the length of the time necessary to count twenty movements. In general the normal mouth movements were about twenty in 11 seconds. On comparing Curves A and B, Fig. 1, it is evident that at these concentrations the decrease in CO_2 output is
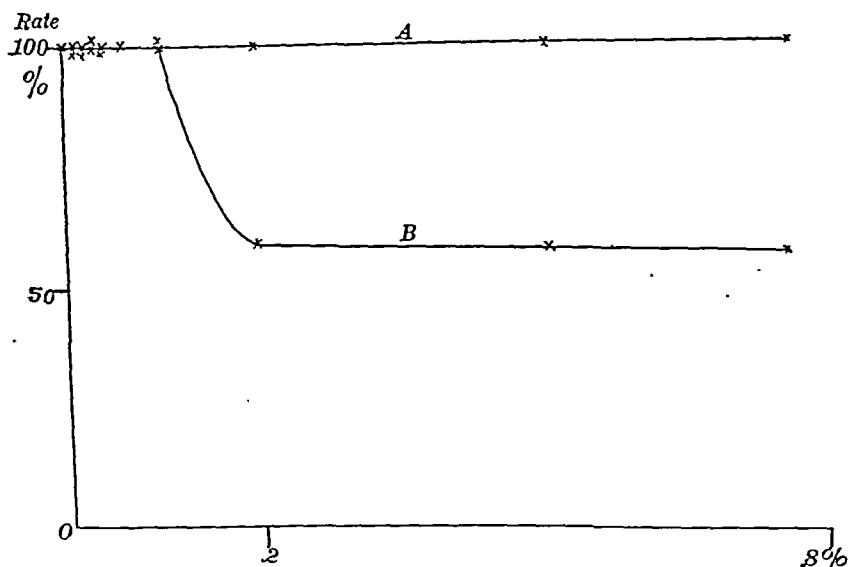
Fig. 1. Curves showing the effect of ether upon the rate of CO_2 production (Curve B) and the rate of mouth movement (Curve A) of frog tadpoles. The normal rate is taken as 100 per cent in each case. The time of exposure to ether was 150 seconds. Average of six experiments. Probable error less than 2.5 per cent of the mean. The figures expressing the per cent of ether should be corrected by multiplying by 0.73.

not due to the decrease in the water supply to the gill system. A quantitative measurement of the body movement was not possible for the reason that the movements of the tail were in many cases too rapid for accurate measurement. However, it may be stated that there was no appreciable decrease in the rate of the body movement at the concentrations in which the retardation of the CO_2 output took place. Thus, in general it may be stated that the de-

crease in CO_2 output takes place in these concentrations before the muscular movements are much affected.

Fig. 2, shows the effect of the 3.65 per cent ether (Curve A) and the effect of the 7.3 per cent ether (Curve B) on the CO_2 output.   With
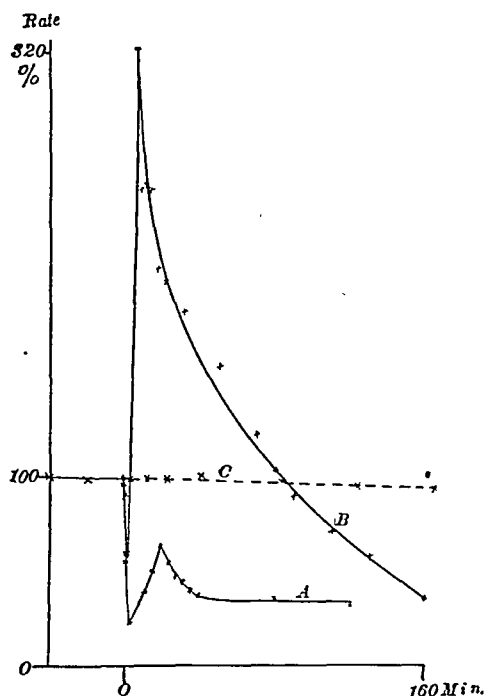
FIG. 2. Curves showing the effect of 3.65 per cent ether (Curve A), and 7.3 per cent ether (Curve B) on the CO_2 production of frog tadpoles, and control in tap water (Curve C, dotted line).   The point marked 0 on the abscissa indicates the beginning of exposure to ether for Curves A and B; previous to this the material was in tap water (horizontal part of curves).   The normal rate (which is taken as 100 per cent) corresponds to a change from pH 8.0 to pH 7.7 in 50 seconds for Curve A, in 43 seconds for Curve B, and in 58 seconds for Curve C.   Each curve represents an average of six experiments.   Probable error less than 2.5 per cent of the mean.

3.65 per cent ether a decrease of 81 per cent took place which was followed by an increase; with 7.3 per cent ether a decrease of 50 per cent occurred which was followed by an increase, the maximum of ac-

celeration being about three times the normal; in both cases decrease takes place after the increase. The reason the initial decrease is less with 7.3 per cent ether than it is with 3.65 per cent, is that the acceleration takes place more rapidly in case of the former, thus preventing the further decrease in CO_2 output.

Both 3.65 and 7.3 per cent ether caused peeling of the cuticular layers of the epidermis; this reaches a maximum in 20 minutes in 3.65 per cent ether, and in 5.5 minutes in 7.3 per cent ether. During the first 4 minutes in 3.65 per cent ether no peeling was observed. It might be supposed that this peeling causes an apparent increase in CO_2 production by allowing the CO_2 to escape more freely. However, the extent of the peeling seems to be the same in both the 3.65 and the 7.3 per cent solutions, although the acceleration in the CO_2 output in the former concentration is much greater than the latter. This difference cannot therefore be wholly due to the peeling of the skin.

Both the breathing movements and the body movements cease at these concentrations in less than 10 seconds after immersion of the tadpoles in the solutions. This might be thought to account for the drop in the rate of CO_2 output, but in view of the fact that we find a similar drop in Fig. 1, where the movement remained normal, we are obliged to conclude that the cessation of the muscular movement cannot account entirely for the falling off in the CO_2 output. This conclusion is confirmed by the experiments on frog eggs.

Aquatic Insects.

In order to avoid the disturbances due to the peeling of the epidermis, an aquatic insect was chosen. This is the common whirligig beetle, *Dineutes assimilis* Aube. It possesses a chitinous covering which prevents any peeling. 7.3 per cent ether solutions were used. Fig. 3, Curve A, shows a 94 per cent decrease in CO_2 output, after which an increase takes place followed again by a decrease.

The activity of these insects is much greater than that of tadpoles and a correspondingly greater drop in CO_2 output is to be expected as the result of anesthesia.
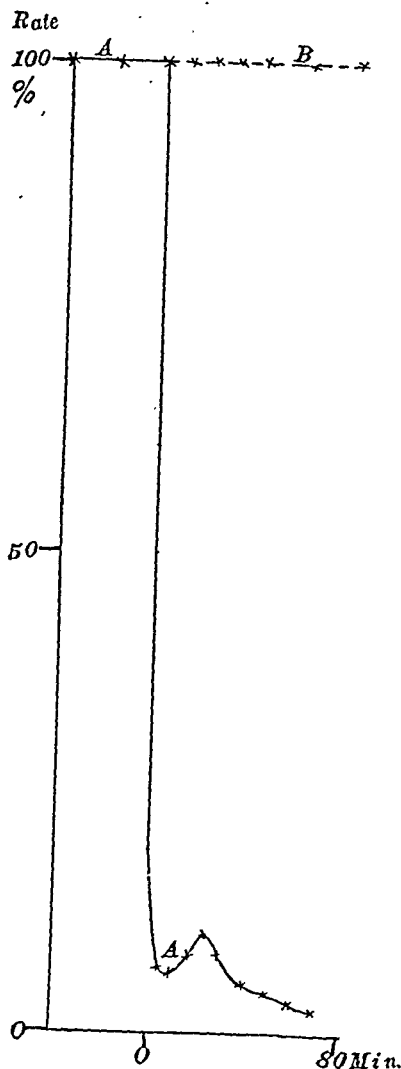
Fig. 3. Curves showing the effect of 7.3 per cent ether (Curve A) on the CO_2 production of an aquatic insect (*Dineutes*), and control in tap water (Curve B, dotted line). The normal rate is taken as 100 per cent. The point marked 0 on the abscissa indicates the beginning of exposure to ether for Curve A; previous to this, the material was in tap water (horizontal part of curves). The normal rate (which is taken as 100 per cent) corresponds to a change from pH 8.0 to pH 7.7 in 38 seconds for Curve A and in 41 seconds for Curve B. Each curve represents an average of six experiments. Probable error less than 2.5 per cent of the mean.

Frog Eggs.

In order to check the above results, frog eggs in the blastopore stage were chosen. The CO_2 output of the jelly (free from any traces of eggs) was first tested. It was found that in the time required for
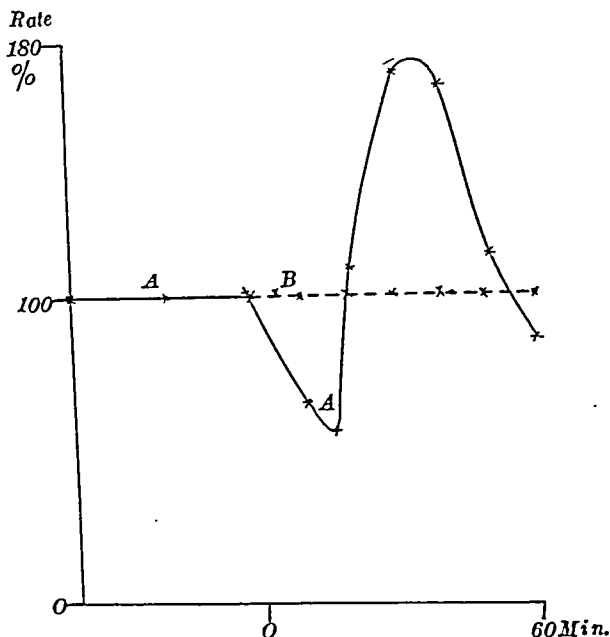
FIG. 4. Curves showing the effect of 7.3 per cent ether (Curve A) on the CO_2 production of frog eggs, and the control in tap water (Curve B, dotted line). The normal rate is taken as 100 per cent. The point marked 0 on the abscissa indicates the beginning of exposure to ether for Curve A; previous to this the material was in tap water (horizontal part of curves). The normal rate (which is taken as 100 per cent) corresponds to a change from pH 8.0 to pH 7.7 in 157 seconds for Curve A and in 142 seconds for Curve B. Each curve represents an average of six experiments. Probable error less than 2.5 per cent of the mean.

the experiments, no appreciable change in the pH value took place, thus proving that the jelly contains no organisms respiring sufficiently to interfere with the accuracy of the experiments. Fig. 4, Curve A, shows a 44 per cent retardation, followed by an increase in the rate of the CO_2 output. There was no disintegration of any portion of the

eggs which might correspond to the peeling of the epidermis in case of the tadpoles, but owing to the presence of cilia, the decrease in CO_2 output may be partly influenced by the cessation of ciliary movement as result of anesthesia.

Fundulus heteroclitus.

In order to understand the true nature of the effect of the production of CO_2 by animals it was necessary to avoid completely the disturbances due to ciliary and muscular movement, and to peeling of the skin. The embryos of the fish, *Fundulus heteroclitus*, 2 days old, were chosen for this purpose; at this stage of embryonic development there are no muscles, no cilia, and no skin, and furthermore the heart beat has not yet commenced. 1 day before the experiment was carried out, the jelly surrounding the membrane was removed, so that the embryos would not adhere to each other during the course of experimentation.

Fig. 5, Curve A, shows the effect of 0.73 per cent ether solution on the CO_2 production of the fish embryos. A gradual decrease takes place to about 46 per cent of the normal, after which the rate remains almost constant during the rest of the experiment. Fig. 5, Curve B, shows the effect of 3.65 per cent ether solution. There is a slight decrease at first, which is followed by an increase of carbon dioxide; this is succeeded by a decrease. Curve C shows the effect of 5.48 per cent ether; a tremendous increase of the carbon dioxide output takes place. If there is any decrease at the start it must be so brief as to escape observation. The rate soon reaches a maximum, after which it falls rapidly.

During the increase in 3.65 per cent and 5.48 per cent ether the embryo becomes gradually more opaque; this process begins to be observable after 20 minutes in 3.65 per cent ether and after 4 minutes in 5.48 per cent ether.

These experiments show that a decrease in the carbon dioxide output may take place as a direct effect of the ether on the protoplasmic substance, when complications due to motion are excluded. This effect may play a part in the phenomena observed in the previous experiments (on tad poles, aquatic insects, and frog eggs) where the cessation of motion is involved.

With tadpoles and *Fundulus* embryos the effect of ether at a concentration of about 0.73 per cent is reversible. The *Fundulus* embryos recover, as shown by their subsequent growth and normal development. The tadpoles also recover, as shown by their subsequent normal respiration. The increase in carbon dioxide production in 3.65
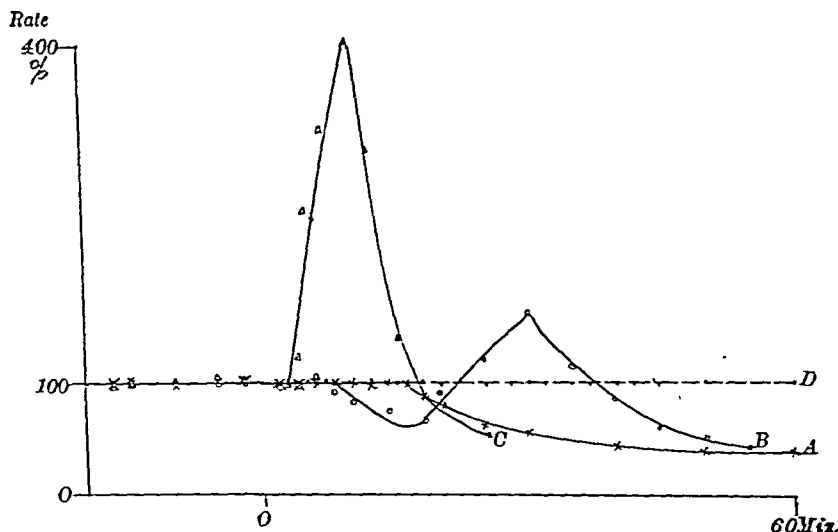
FIG. 5. Curves showing the effect of 0.73 per cent ether (Curve A), 3.65 per cent ether (Curve B), and 5.48 per cent ether (Curve C), on the CO_2 production of *Fundulus* embryos, and control in tap water (Curve D, dotted line). The point marked 0 on the abscissa indicates the beginning of exposure to ether for Curves A, B, and C; previous to this, the material was in tap water (horizontal part of curves). The normal rate (which is taken as 100 per cent) corresponds to a change from pH 8.0 to pH 7.7 in 128 seconds for Curve A, in 119 seconds for Curve B, in 176 seconds for Curve C, and in 111, seconds for Curve D. Each curve represents an average of six experiments. Probable error less than 2 per cent of the mean.

and 7.3 per cent ether is accompanied by irreversible changes leading to death. If *Fundulus* embryos were removed from the ether about 75 seconds after the increase began in 3.65 per cent ether, they failed to recover. In tadpoles the recovery is no longer possible 47 seconds after the increase has begun. In frog eggs similar results were obtained.

It is evident from these experiments that in animals the effect of ether is of two types: (1) the decrease in the output of carbon dioxide, which is reversible; (2) the increase of the output of CO_2 which is irreversible.

It was shown by Loeb and Wasteneys[4] that 1 per cent ether produces narcosis in the 1 week old embryos of *Fundulus*, and there can be little question that this is also the case in embryos used in my experiments. The question arises whether the decrease in the output of carbon dioxide observed in 0.73 per cent ether is sufficient to produce narcosis. In order to test this, experiments were made to determine to what degree the temperature must be lowered in order to reduce carbon dioxide output of 2 day embryos to the same point as in 0.73 per cent ether (that is, to 57 per cent of the normal). It was found that lowering the temperature from 22 to 13°C. produced the desired result. But it was observed that no narcosis was produced by this drop in temperature, for when 8 day embryos were similarly treated, their movements remained practically normal. We must therefore conclude, as did Loeb and Wasteneys[5] from an experiment on sea urchin eggs, that the decrease in respiration is wholly inadequate to produce narcosis. It is therefore evident that the action of anesthetics producing narcosis is due to some other cause than the effect upon respiration. This is in complete agreement with the striking experiments of Loeb and Wasteneys[4] on *Fundulus* embryos, where they found that oxidation must be reduced to one-fourteenth of the normal in order to produce narcosis.

A comparison of the data obtained by the writer on the effect of ether on the carbon dioxide production of tadpoles, aquatic insects, frog eggs, and killifish eggs with those described in the preceding articles of this series, as well as with those obtained by Haas,[6] shows that there exists a difference in plants and animals as regards the effect of ether. With animals, in weaker concentrations, such as 0.73 per cent solution of ether, a decrease in the CO_2 output takes place, and the effect is reversible, while in stronger concentrations, such as 3.65

[4] Loeb, J., and Wasteneys, H., *Biochem. Z.*, 1913, lvi, 295.
[5] Loeb and Wasteneys, *J. Biol. Chem.*, 1913, xiv, 517.
[6] Haas, *Science*, 1917, xlvi, 462.

per cent and 7.3 per cent ether, an increase in the carbon dioxide production takes place, the effect being irreversible. This is different from the effects usually found in plants.

SUMMARY.

1. The experiments on frog tadpoles show that with 0.15, 0.37, and 0.55 per cent ether solutions there is a decrease in CO_2 output. The effect is reversible. With these concentrations the breathing movements and body movements remained normal during the experiment. In 3.65 and 7.3 per cent ether there is a decrease of respiration followed by an increase which in turn is followed by a decrease. The increase may reach about three times the normal rate. The increase in the CO_2 output is accompanied by the peeling of the skin. The effect is irreversible.

2. Experiments on an aquatic insect, *Dineutes assimilis* Aube, show that in 7.3 per cent ether there is a decrease followed by an increase which in turn is followed by a decrease. There is no apparent disintegration of structures in the organism accompanying the increase. The effect is irreversible.

3. The experiments on frog eggs with 7.3 per cent ether show a result similar to that found in aquatic insects.

4. Experiments on *Fundulus* embryos show that with 0.73 per cent ether there is a reversible decrease in the rate of CO_2 production. In 3.65 per cent ether there is a temporary decrease followed by an increase, after which the rate begins to fall off. In 7.3 per cent ether there is an immediate increase amounting to 307 per cent which is followed by a decrease. The increase in the 3.65 and 7.3 per cent ether is accompanied by irreversible changes leading to death. The decrease found in 0.73 per cent ether is not sufficient to cause narcosis, as is shown by experiments on which the same decrease is produced by lowering the temperature.

5. These experiments show that narcosis is not due to asphyxia. The action of anesthetics is due to some other cause than the effect on respiration.

There is a difference between the animals studied and the plants described in this series of articles, since in animals the increase in the

CO_2 output is accompanied by irreversible changes leading to death, while this is not necessarily the case in plants. The reversible (narcotic) action of ether on the animals studied was accompanied by a decrease in the carbon dioxide output; in plants this is not ordinarily the case.

These facts are of considerable interest, but their interpretation must be left to future investigation.

ON THE CONTROL OF ROPE IN BREAD.*

By E. J. COHN,

First Lieutenant, Sanitary Corps, U. S. Army,

S. B. WOLBACH, L. J. HENDERSON,

AND P. H. CATHCART,

First Lieutenant, Sanitary Corps, U. S. Army.

(From the Wolcott Gibbs Memorial Laboratory of Harvard University and the Pathological Laboratory of the Peter Bent Brigham Hospital, Boston.)

Rope is a condition of bacterial decomposition of bread which leads to a peculiar slimy or ropy consistency of portions of the interior of the loaf, produces an odor resembling that of ensilage, and renders the loaf unacceptable. During summer months the loss of bread through the development of rope may be very great, and individual bakeries may become so thoroughly infected that they can be operated only with great difficulty.

Several closely related organisms have been isolated from ropy bread, some or all of which are undoubtedly the effective agents of the decomposition.[1-3]

All varieties of bread appear to be liable to this decomposition within 24 hours or more after baking, but it has been stated that those containing a considerable quantity of material other than white flour are most often infected. For many years it has been customary to add lactic acid, acetic acid, or some other acid substance to the dough as a means of repressing the growth of the organism. Under favorable circumstances this method of treatment may be very satisfactory; but it is always carried out by rule of thumb, and there is at present

* Published with the permission of the Surgeon General.

[1] Lafar, F., Handbuch der technischen Mykologie, Jena, 1905–08, ii, 520.

[2] Fuhrmann, F., Vorlesungen über technische Mykologie, Jena, 1913, 257; *Centr. Bakteriol., 2te Abt.*, 1906, xv, 385, 538.

[3] Beattie, J M., and Lewis, F. C., *Lancet*, 1917, ii, 211

no general agreement as to the kind or quantity of acid that should be employed, or the scientific explanation of its action.

The present investigation has been upon a strain of *Bacillus mesentericus* isolated from a ropy loaf and characterized by one of us. In addition three other cultures, from two other sources, have been examined and appear to be identical.

The bacteriological study of the bacillus, which belongs to the *Bacillus mesentericus* group, is not completed and there has been no opportunity for comparison with cultures from other sources. Since a number of bacteria, all probably closely related, have been described as causes of ropy bread and since the bacteriological data are meager and not recent, it seems best, for the present, not to name the organism isolated in this vicinity. Its relationship to the production of characteristic rope has been proved beyond question, and the four cultures from three different local sources are identical.

The bacillus is a motile, Gram-positive, spore-bearing bacillus. In 24 hour cultures on plain agar its dimensions average 2.5 microns by 0.5 to 0.75 microns. The ends of the rods are rounded. The spores are ovoid in shape and are situated approximately in the middle of the bacilli. The width of the spores slightly exceeds that of the rods and they measure 0.5 to 1.75 microns by 0.75 to 0.85 microns. The spores are produced in great abundance in cultures less than 24 hours old and are extremely resistant to boiling.

The bacillus is a strict aerobe. It grows readily on all ordinary media, resulting in a decrease of acidity. Coagulated blood serum is completely liquefied. Litmus milk is rapidly curdled and liquefied. The litmus is reduced in the depths of the medium but at the surface the color becomes deeper blue. Cultures in agar are odorless, but in wheat flour media the characteristic odor of ropy bread is produced.

The colonies on agar media are flat, finely granular, dull white or grayish, with irregular margins. In 24 hours the colonies become wrinkled or thrown into coarsely irregular elevations, and the margins become convoluted or arborescent. Colonies several days old develop a brownish tint which is more marked upon media containing carbohydrates. On liquid media a wrinkled and convoluted pellicle is formed; turbidity develops slowly, and begins beneath the surface pellicle.

The optimum temperature is about 38°C. At ordinary room temperatures (20–24°C.) growth is very slight.

The action on bread was studied by the inoculation of baked loaves and by the incorporation of cultures in the dough. In both cases the bread was incubated at 37.5°C. and examined after 24 or 36 hours. The rope produced by addition of the pure cultures was identical in physical properties and odor with that of the bread from which the cultures were isolated.

In all instances the softening occurred just in the centers of the loaves and progressed outwards. The foci of softening in bread took on a brownish tint and became sticky, and particles on separation yielded delicate threads of from a few inches to 6 or 7 inches in length. In the case of cultures introduced with considerable amounts of liquid media a cavity was produced. In bread or dough to which acid was added the softening and odor were less pronounced and were finally suppressed at a hydrogen ion concentration of approximately $10^{-5}N$. In acidities sufficient to retard the growth of the organism the discoloration of the softened foci did not occur.

The original loaf was softened and discolored, and slowly developed the characteristic slimy and viscous condition. Examination of an aqueous extract of a decomposed portion of this loaf with the concentration cell revealed a marked diminution of acidity,—from approximately pH 5.3, characteristic of normal bread, to pH 6.2. Other specimens gave the following values for pH; 6.5, 6.1, 6.3.

As a result of these observations we were led to a study of the relation between hydrogen ion concentration and the growth of the organism.

Three culture media were prepared: a synthetic medium known as Cohn's solution, a potato medium, and a wheat paste. These media were then divided into portions, acid or alkali was added to bring these to different hydrogen ion concentrations, they were sterilized, and finally inoculated with a culture of the organism, or of the spores.

The accompanying curves of the variation in hydrogen ion concentration of these media during titration will indicate the general characteristics of the preparations, and may perhaps serve others as a basis for similar work.
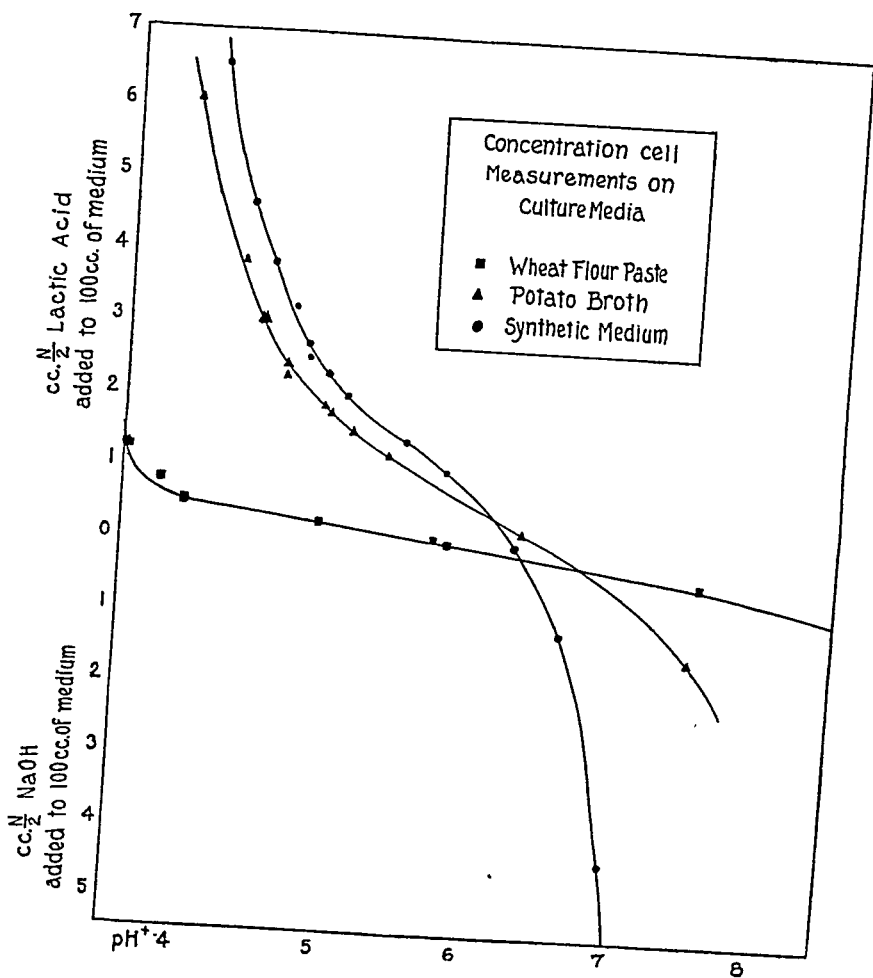
FIG. 1.

Preparation of the Media.

Cohn's Medium with the Addition of Asparagine.

K₂HPO₄	5.0 gm.
Ca₃(PO₄)₂	0.5 "
MgSO₄	5.0 "
Ammonium tartrate	10.0 "
Asparagine	4.0 "
Distilled water	1,000.0 cc.

The ingredients were weighed and dissolved in the water and after tubing were sterilized in the autoclave at 15 pounds pressure for 30 minutes.

Wheat Bouillon.

Wheat flour	15.0 gm.
MgSO₄	0.5 "
KNO₃	1.0 "
Dextrose	15.0 "
Distilled water	1,000.0 cc.

The wheat flour was added after solution of the other ingredients and the whole boiled for a few minutes, tubed, and sterilized in the autoclave at 15 pounds pressure for 30 minutes.

Potato Broth.—Two parts of water to one of freshly grated raw potatoes were mixed and allowed to stand for 24 hours. The mixture was then strained through cloth and the liquid filtered through coarse filter paper, tubed, and sterilized at 15 pounds pressure for 30 minutes. It was not considered of importance to avoid the precipitate which occurred.

The cultures were incubated for 5 days; meanwhile they were observed daily, and finally both inoculated tubes and controls were examined for their hydrogen ion concentrations. The results of this experiment are presented in Table I. It should be noted that the results with the wheat paste were uncertain. This appears to be due to its lack of homogeneity, and low buffer content. It is also to be observed that the spores are, as might be expected, somewhat more restricted in growth than the organisms themselves.

A second experiment was performed over a narrower range of hydrogen ion concentration, the results of which, presented in Table II, agree satisfactorily with those of the first experiment.

From these experiments it seems safe to conclude that a hydrogen ion concentration sensibly higher than $10^{-5}N$ completely inhibits the growth of *Bacillus mesentericus*.

TABLE I.

Growth of Rope at Different Hydrogen Ion Concentrations.

Experiments 668 and 685.

Tube No.	Solution added to 100 cc. of medium.	Organisms: Day 1st	2nd	3rd	4th	5th	Spores: Day 1st	2nd	3rd	4th	5th	pH after incubation Control.	Organisms.	Spores.
					Wheat flour paste.									
1	6.0 0.1 N C₃H₆O₃ (cc.)	0	0	0	0	0						3.53		
2	4.0 0.1 " "	0	0	0	0	0						3.77	3.63	
3	2.4 0.1 " "	0	0	0	0	0						(5.22)	3.94	
4	1.3 0.1 " "	0	+	+	+	+	0	?	+	+	+	4.87	4.40	5.45
5	0.4 0.1 " "	0	+	+	+	+	0	+	+	+	+	5.68	6.62	5.47
6	0.3 0.1 " NaOH	0	++	+++	+++	+++	0	+	+	+	+	5.82	5.58	5.80
7	0.8 0.1 " "	0	++	+++	+++	+++	0	+	++	++	+++	6.05	5.41	5.73
8	1.4 0.1 " "	0	++	+++	+++	+++	0	+	++	++	+++	5.99	5.16	5.54
9	2.7 0.1 " "	0	++	+++	+++	+++	0	+	++	++	+++	5.92	5.06	5.83
10	4.4 0.1 " "	0	+	+	+	+	0	+	+	+	++	5.60	5.49	5.88
					Cohn's solution.									
12	12.8 0.5 N C₃H₆O₃	0	0	0	0	0	0	0	0	0	0	3.82	3.75	
13	6.5 0.5 " "	0	0	0	0	0	0	0	0	0	0	4.07	4.12	
14	2.5 0.5 " "	0	+	++	++	++	0	0	0	0	0	4.75	4.72	4.77
15	1.0 0.5 " "	0	++	+++	+++	+++	0	0	0	0	0	5.74	6.92	5.46
16	0	0	++	+++	+++	+++	0	0	0	0	0	6.25	7.08	6.23
17	1.2 0.5 " NaOH	0	0	++	+++	+++	0	0	++	++	++	6.60	7.74	6.39
18	4.4 0.5 " "	0	0	0	++	++	0	+	++	++	++	6.98	7.94	7.93
19	9.1 0.5 " "	0	0	0	+	++	0	+	+	+	++	7.14	8.11	8.17
20	11.2 0.5 " "	0	0	0	0	+	0	+	+	+	+	7.64	8.21	8.20

TABLE I—*Concluded.*

Potato broth.

22	12.0 0.5 N C₃H₆O₃	o	o	o	o	o	o	o	o	o	3.39	3.53	
23	6.0 0.5 " "	o	o	o	o	o	o	o	o	o	3.90	3.94	
24	4.0 0.5 " "	o	o	o	o	o	o	o	o	+	4.09	4.08	4.19
25	3.0 0.5 " "	o	+	+	+	+	+	+	+	+	(4.52)	4.40	4.39
26	2.25 0.5 " "	o	+	+	+	+	+	+	+	+	4.59	7.01	4.68
27	1.75 0.5 " "	+	+	+	+	+	+	+	+	+	4.92	6.63	7.37
28	1.20 0.5 " "	+	+	+	+	+	+	+	+	+	4.93	7.10	7.30
29	0.20 0.5 " "	+	+	+	+	+	+	+	+	+	6.28	7.05	7.60
30	1.50 0.5 " NaOH	+	+	+	+	+	+	+	+	+	7.49	7.57	7.65

In order to test this conclusion upon bread itself a series of loaves of different hydrogen ion concentrations were prepared with the co-operation of Messrs. C. F. Hathaway and Sons of Cambridge.   These

TABLE II.

Growth of Rope at Different Hydrogen Ion Concentrations.

Experiments 686 and 700.

Tube No.	Solution added to 100 cc. of medium.	Organisms: Day.					Spores: Day.					pH after incubation.		
		1st	2nd	3rd	4th	5th	1st	2nd	3rd	4th	5th	Control.	Organisms.	Spores.
	Wheat flour paste.													
	cc.													
1	1.4 0.1 N C₃H₆O₃	+	+	++	++	++	0	+	+	+	+	5.12	5.78	5.57
2	1.15 0.1 " "	+	+	++	++	++	0	+	+	+	+	5.22	5.55	6.78
3	0.90 0.1 " "	+	+	++	++	++	0	++	++	++	++	5.66	5.75	5.78
4	0.70 0.1 " "	+	++	++	++	++	0	+	+	+	+	5.64	5.97	5.97
5	0.55 0.1 " "	+	++	++	++	++	0	+	+	+	+	6.40	5.68	6.63
6	0.45 0.1 " "	+	++	++	++	++	0	+	+	+	+	6.30	5.71	6.43
	Cohn's solution.													
12	4.6 0.5 N C₃H₆O₃	0	0	0	0	0	0	0	0	0	0	4.30	4.33	4.34
13	3.8 0.5 " "	0	0	0	0	0	0	0	0	0	0	4.46	4.46	4.46
14	3.2 0.5 " "	+	+	+	+	+	0	0	0	0	0	4.63	4.61	4.61
15	2.7 0.5 " "	+	++	++	++	++	0	0	0	0	0	4.73	6.84	4.73
16	2.3 0.5 " "	0	0	0	0	0	0	0	0	0	0	4.89	4.91	4.90
17	2.0 0.5 " "	+	++	++	++	++	0	0	0	0	0	5.03	7.19	5.03
18	1.4 0.5 " "	+	+	+	+	+	0	0	0	0	0	5.45	7.39	5.50
	Potato broth.													
21	3.8 0.5 N C₃H₆O₃	0	0	0	0	0	0	0	0	0	0	4.27	4.25	4.25
22	3.0 0.5 " "	0	0	0	0	0	0	0	0	0	0	4.42	4.43	4.41
23	2.4 0.5 " "	0	0	0	0	0	0	0	0	0	0	4.59	4.64	4.61
24	1.85 0.5 " "	0	+	++	++	++	0	0	++	++	++	4.85	6.59	6.51
25	1.50 0.5 " "	0	+	++	++	++	+	+	+	+	+	5.07	6.33	7.25
26	1.20 0.5 " "	+	++	++	++	++	+	+	+	+	+	5.28	7.52	7.24

loaves were then inoculated with a culture of the organism and incubated in a moist condition at a temperature of 37.5°C.   The results of this experiment are presented in Table III.

TABLE III.

Growth of Rope in Bread. Inoculation After Baking.

Loaf No.	pH	Growth of organisms.
12	4.31	0
9	4.55	0
5	4.77	0
8	4.81	+
10	4.87	0
11	5.07	+
6	5.09	+
7	5.25	+
1	5.28	0*
2	5.35	++
3	5.44	++
4	5.47	++

* Large buffer content.

In order to reproduce more closely the actual conditions of baking another series of loaves were baked in our own laboratory from dough to which 1 cc. of a culture of the organism per loaf of bread had been added. The results of this experiment are given in Table IV.

TABLE IV.

Growth of Rope in Bread. Inoculated Before Baking.

Loaf No.	0.5 N lactic acid added to dough.	pH		Growth.
		Before baking.	After baking.	
	cc.			
1	0	5.30	5.38	++
2	1.0	5.24		+
3	1.6			+
4	2.4			?
5	3.2	4.98		0
6	4.0	4.94	4.98	0

Taking account of these facts and of the observation which we have made in another connection, that wheat substitutes are commonly less acid than wheat flour and often contain larger amounts of buffer substances, it seems safe to conclude, first, that the development of

animal contains a nerve net and hence may serve as a region in which to measure the rate of transmission in that type of nervous tissue.

The method employed in this procedure was that long since used in the measurement of nerve rates. A stimulus was applied to the distal end of the tongue (Fig. 1, *2*), and the time intervening between the moment of application and the moment of response was measured. The stimulus was next applied at the root of the tongue (Fig. 1, *3*), and the time between the moment of application and the moment of response was again measured.   The difference between the amounts of time consumed in these two operations represents the time necessary for the transmission of an impulse from the distal end of the tongue
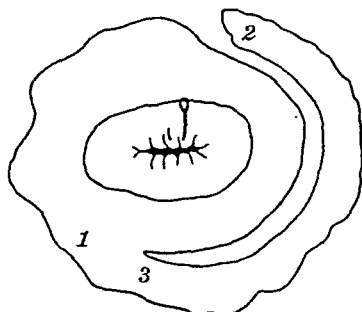

FIG. 1. Oral view of a *Metridium* showing the tongue of tissue cut from the pedal margin of the column, the three points of stimulation, *1, 2,* and *3,* and the region of the lip into which the hook was inserted.

to its root, a distance that is easily measured and that thus affords a basis for the determination of a rate.

The records of the several operations necessary in these measurements were made in the usual way on a kymograph.   Time was recorded by a marker actuated by an electrically driven tuning fork at the rate of 50 vibrations per second.   The moment of applying the stimulus was recorded by a second marker which was activated electrically from the same switch through which the stimulating current was sent.   This consisted of a faradic current that was disagreeably strong to the human finger.   It was applied by appropriate electrodes to the portion of the pedal edge of the sea anemone that was to be stimulated.   This edge, as is well known, is extremely

sensitive to mechanical contact but only moderately so to electrical stimulation. It was, therefore, important to place the electrodes in position without disturbing the animal and to stimulate with the electric current only after all possibility of mechanical disturbance had been passed. The response of the animal was recorded on the drum of the kymograph by a delicate heart lever which was attached by a thread and hook to the lip of the sea anemone. As a matter of good fortune the lip of *Metridium* is extremely insensitive and a hook may be passed through it and drawn up lightly without calling
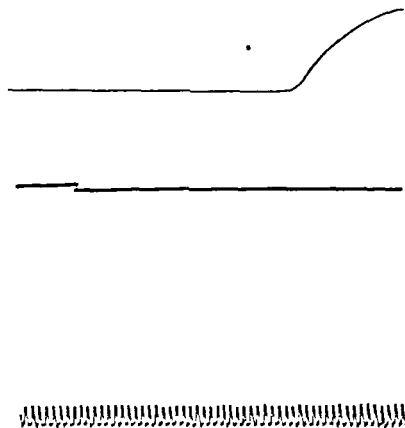

FIG. 2. Kymograph record in which the lowest line represents time in fiftieths of a second, the middle line the moment of application of the stimulus, and the uppermost line the response of the sea anemone. The stimulus was applied at position *1* (Fig. 1).

forth any particular response on the part of the animal. With this attachment the slightest contraction of the longitudinal muscles of the mesenteries was easily observed.

Large specimens of *Metridium* were allowed to attach themselves to sheets of glass about 15 cm. square. After they had firmly fixed themselves, each one was subjected to the following procedure. After the necessary connections between the animal and the kymograph were made, an electric stimulus was applied to the pedal edge of the column and the normal reaction time, as indicated by the retraction of the oral disc, was determined (Fig. 2). The long tongue

of tissue was then cut from the pedal edge (Fig. 1). After the sea anemone had reexpanded, the stimulus was applied to the distal end of this tongue and the time between the moment of application of
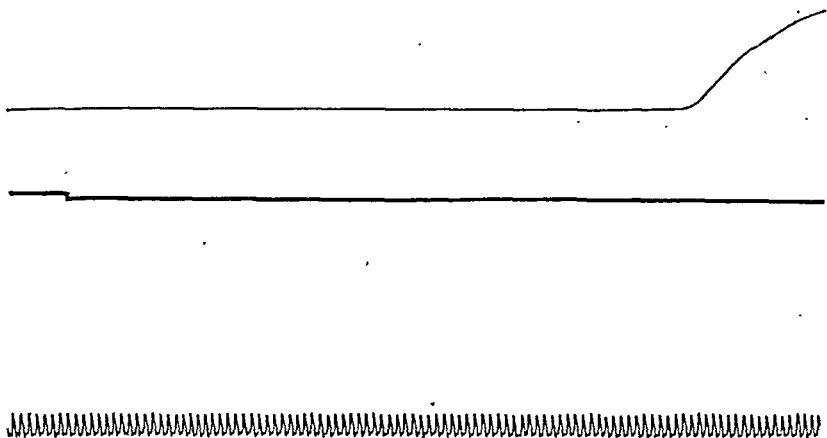
FIG. 3. Kymograph record. Stimulus applied at position *2* (Fig. 1).

FIG. 4. Kymograph record. Stimulus applied at position *3* (Fig. 1). This record is from a set not included in Table I.

the stimulus and the moment of response was determined (Fig. 3). The stimulus was next applied to the base of the tongue and a second determination was made (Fig. 4). Three sets of these three deter-

minations were made from each animal. After these steps had been taken the glass plate to which the sea anemone was attached was turned over and the length of the tongue from one point of stimulation to the other was measured. In preparing the tongue it was found easy to leave the tissue attached to the glass plate to which the sea anemone as a whole had attached itself and thus to facilitate greatly the measurement of the length of the tongue. The results of these measurements on three animals, A, B, and C, are given in Table I.

TABLE I.

Reaction times are expressed in hundredths of a second of three specimens of *Metridium*, A, B, and C, to faradic stimuli applied to the pedal edge of the column at points *1*, *2*, and *3* (Fig. 1) and responded to by the contraction of the longitudinal mesenteric muscles. The readings were made in fiftieths of a second, but for convenience in tabulation they have been converted into hundredths of a second. The rates of transmission, as calculated from data contained in the table, are given for the three animals in the lowest line in the table. During experimentation the animals were kept in sea water at a temperature of 21°C.

Specimen.	A		B		C	
	Observations.	Averages.	Observations.	Averages.	Observations.	Averages.
	sec.	*sec.*	*sec.*	*sec.*	*sec.*	*sec.*
Reaction time from point *1*.	0.60 0.62 0.58	0.600	0.64 0.62 0.66	0.640	0.56 0.52 0.58	0.553
Reaction time from point *2*.	1.48 1.36 1.64	1.493	1.98 1.66 2.04	1.893	1.40 1.50 1.46	1.453
Reaction time from point *3*.	0.56 0.68 0.60	0.613	0.66 0.58 0.68	0.640	0.54 0.60 0.50	0.547
Time between points *2* and *3, sec.*		0.880		1.253		0.906
Distance from point *2* to point *3, mm.*	119		152		132	
Rate per sec., *mm.*	135+		121+		146−	

In Specimen A the average reaction time over the tongue of tissue was found to be 1.493 seconds and from the root of the tongue 0.613 seconds, which agrees very closely with that of the intact animal, 0.60 seconds.   The time consumed in transmission over the tongue is the difference between 1.493 and 0.613 or 0.880 seconds.   As the length of the tongue between the points stimulated was 119 mm., it follows that the rate of transmission was a little over 135 mm. per second.   By a similar method of procedure the rate in Specimen B was determined to be 121 mm. per second, and in Specimen C 146 mm. per second.

These rates are relatively low compared with even the lower rates already determined for transmission in the nerve fibers of metazoans, but this is not surprising, for the nerve net has generally been supposed to be a less rapid and efficient organ of transmission than the nerve trunk.   The rate is lower than that given by Romanes (1878) for what he calls the stimulus wave in *Aurelia*, namely 9 inches or about 229 mm. per second, and is much lower than that given by Harvey (1912) for the contraction wave in *Cassiopea*, some 775 mm. per second.   In both these instances, however, the rates measured refer to waves of muscular activity and though these waves very probably reflect accurately the rate of nervous transmission, they are not free from complications with possible muscular transmission, whereas the rates derived from *Metridium* are.   The measurements taken on *Cassiopea* were made at Dry Tortugas, Florida, where the sea water is relatively warm, a condition which, as is well known, increases the rate of transmission.   At Woods Hole, Massachusetts, where the measurements on *Metridium* were made, the sea water in which the sea anemones were immersed during experimentation had a temperature of 21°C.

CONCLUSION.

Nerve net transmission in *Metridium* at 21°C. varies from 121 to 146 mm. per second.

BIBLIOGRAPHY.

Harvey, E. N., 1912, The permeability of living cells for alkalies.   The question of nerve fatigue, *Carnegie Institution of Washington, Year Book, No. 10*, 130–131.

Romanes, G. J., 1878, Further observations on the locomotor system of medusæ, *Phil. Tr. Roy. Soc.*, clxvii, 659–752.

AMPHOTERIC COLLOIDS.

II. VOLUMETRIC ANALYSIS OF ION-PROTEIN COMPOUNDS; THE SIGNIFICANCE OF THE ISOELECTRIC POINT FOR THE PURIFICATION OF AMPHOTERIC COLLOIDS.

BY JACQUES LOEB.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

INTRODUCTION.

In order to understand the influence of the hydrogen ion concentration upon the chemical and physical behavior of proteins we may well keep in mind the rôle of the hydrogen ion concentration upon the reactions of an amphoteric metal hydroxide, like aluminium or chromic hydroxide, whose chemical constitution is better known than that of the proteins.

"Aluminium hydroxide dissolves in acids. From its solution in hydrochloric acid, an aluminium salt, aluminium chloride $AlCl_3 \cdot 6H_2O$. is obtained. It also combines with strong bases, dissolving for instance in a solution of sodium hydroxide and forming an aluminate, $NaAlO_2$.... According to the best knowledge we have on the subject, the molecule of aluminium hydroxide has the following structure or arrangement of its atoms $Al(-O-H)_3$. It is readily seen that the cleavage of the molecules may produce, either aluminium and hydroxide ions, characteristic ions of a base, or aluminate and hydrogen ions, characteristic ions of an acid:

$$Al^{+++} + 3\,\overline{OH} \rightleftharpoons Al(-O-H)_3 \rightleftharpoons Al\overline{O}_2 + H^+ + H_2O."[1]$$
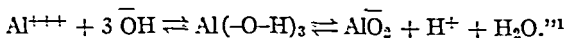
It is obvious that between the action of acids and of bases producing these two types of electrolytic dissociation there must be one hydrogen ion concentration in which aluminium hydroxide is practically neither able to form Al^{+++} nor $Al\overline{O}_2$ ions, and this would be the isoelectric point.

---

[1] Stieglitz, J., The elements of qualitative chemical analysis, New York, 1911, 171.

We may apply the experience gained from aluminium hydroxide to proteins which are also amphoteric electrolytes and for some of which the exact location of the isoelectric point is known. For gelatin it lies at pH = 4.7. In a previous paper[2] it was shown that when pH > 4.7, gelatin is only able to dissociate as gelatin (capable of combining with metals to form metal gelatinates, *e.g.* sodium gelatinate); when pH < 4.7, gelatin is only capable of dissociating as gelatin (capable of combining with anions to form, *e.g.*, gelatin chloride). At the isoelectric point it is practically not dissociated at all. There is this difference between amphoteric electrolytes of the type of proteins and that of metal hydroxides, namely that in the case of proteins the OH ions and the H ions are attached to different groups in the molecule.

We also have shown that a metal gelatinate must give off its metal ion when the hydrogen ion concentration is raised beyond that of the isoelectric point; and that a gelatin-anion compound must give off its anion when the hydrogen ion concentration is lowered below that of the isoelectric point. At the isoelectric point gelatin cannot be in combination with either anion or cation and such gelatin free from *ionogenic* impurities we designate as pure gelatin. It would thus appear that we can free proteins and amphoteric colloids in general from *ionogenic* impurities by bringing the ampholyte to its isoelectric point. Such impurities, however, as form part of the complex gelatin ion can, of course, not be removed by this method. In this and some following papers we intend to furnish additional proof for the correctness of these ideas. This can be done by making the gelatin combine with such ions as Ag or Br or CNS, etc., whose quantity can be easily determined volumetrically.

The Combination of Cation with Gelatin.

In all experiments in which the chemical or physical influence of an electrolyte upon an amphoteric colloid is to be investigated it is necessary to remove the excess of electrolyte after it has had time to act on the colloid; and the writer has shown that the neglect of this precaution has caused a good deal of the confusion which prevails in the

[2] Loeb, J., *J. Gen. Physiol.*, 1918, i, 39.

literature of colloid chemistry. By using gelatin in the condition of a fine powder we are able to wash away the excess of electrolyte after it has had time to act on the gelatin.

When we put 1 gm. of powdered gelatin for 30 minutes at 20 or 15° into 100 cc. of 3 $\text{m}/1024$ HCl or HNO$_3$, and then wash the gelatin on the filter to remove the excess of acid, we obtain a gelatin with pH of about 4.7; $i.e.$, of the isoelectric point. When we use a higher concentration than 3 $\text{m}/1024$ HCl, we get a pH < 4.7, and a gelatin which is on the acid side of the isoelectric point and which is able to act only as cation; when we use an acid less concentrated than 3 $\text{m}/1024$, we get after the excess of acid is washed away a gelatin whose pH > 4.7 and which being on the alkaline side of its isoelectric point can act only as an anion and can combine only with a cation.

In order to get clear results it is well to work with a series of different concentrations of an acid, $e.g.$ $\text{m}/4$, $\text{m}/8$, $\text{m}/16$, etc., to $\text{m}/8192$ HCl or HNO$_3$, so that we obtain series of gelatin solutions with different pH on either side of the isoelectric point. After the excess of acid is washed away by several perfusions with H$_2$O, the gelatin is perfused three times with 25 cc. of the same solution of a salt, $e.g.$ $\text{m}/8$ NaBr or $\text{m}/8$ NaCNS or $\text{m}/16$ AgNO$_3$, to bring about a reaction between salt and gelatin. It is necessary to stir up the gelatin on the filter while the salt solution is poured on to bring about as complete a contact between the powdered particles of gelatin and salt as possible. After the salt solution has drained off, the excess of salt is washed away by six perfusions with 25 cc. of H$_2$O each. The gelatin is then melted and made into a 1 per cent solution whose pH is determined and which is then analyzed for Br or silver, as the case may be. We shall describe some of the results obtained by this method.

1 gm. of finely powdered gelatin is put for 30 minutes at 15°C. into each of a series of beakers containing 100 cc. of HNO$_3$ varying from $\text{m}/8$ to $\text{m}/8192$; and into one beaker containing 100 cc. of distilled water serving as a control. The gelatin is then put on a filter, washed, and is perfused three times with 25 cc. of $\text{m}/16$ AgNO$_3$ in the way described, and subsequently six times with 25 cc. of H$_2$O to wash away the excess of AgNO$_3$. The swelling is measured while the gelatin is still in the cylindrical funnel, and the gelatin is melted and made into a 1 per cent solution. This part of the experiment is of course carried

out in a dark room. Then the pH is determined, and 25 cc. of the solution are used to determine the quantity of silver in combination with the gelatin, according to Volhard's method.

Fig. 1 contains two curves the abscissæ of which are the logarithms of the concentrations of HNO_3 with which the gelatin was originally
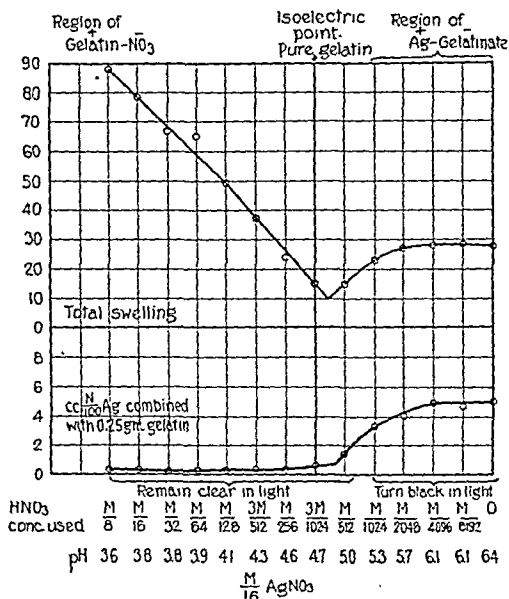

FIG. 1. Gelatin treated with different concentrations of HNO_3, from $M/8$ to $M/8192$, washed, and then treated with the same concentration of $AgNO_3$ ($M/16$), and then washed again. Abscissæ show concentrations of acid used. The final pH of the gelatin solution is found under the figure for the concentration of acid used.

The ordinates of the lower curve give the values for the silver found in combination with the gelatin. The curve shows that at the isoelectric point (pH = 4.7) and on the acid side of the isoelectric point, the gelatin was practically free from silver. On the more alkaline side the amount of silver found in combination with the gelatin increased with the pH. This proves that gelatin can combine with a cation only on the alkaline side from the isoelectric point, and this is corroborated by the fact that on the alkaline side from the isoelectric point only was the gelatin darkened by light. The ordinates of the upper curve are the values for the swelling of the same gelatin. On the alkaline side from the isoelectric point, where the gelatin had combined with silver, the curve for swelling runs parallel to the curve for silver gelatinate formed. It was, therefore, the relative mass of silver gelatinate formed which determined the physical properties of gelatin.

treated. Under each concentration is put the pH found for the gelatin solution at the end of the experiment. The ordinates of the lower · curve are the numbers for the cc. of 0.01 N silver in combination with 0.25 gm. of gelatin. The curve shows that at the isoelectric point (pH = 4.7) the gelatin is free from ionic silver and that the same is true for all the gelatin on the acid side from the isoelectric point (pH 3.6 to 4.7). On the alkaline side from the isoelectric point the

| $\frac{M}{32}$ | $\frac{M}{64}$ | $\frac{M}{128}$ | $\frac{3}{512}$ | $\frac{M}{256}$ | $\frac{3}{1024}$ | $\frac{M}{512}$ | $\frac{M}{1024}$ | $\frac{M}{2048}$ | $\frac{M}{4096}$ | $\frac{M}{8192}$ | $_{H_2}O$ |

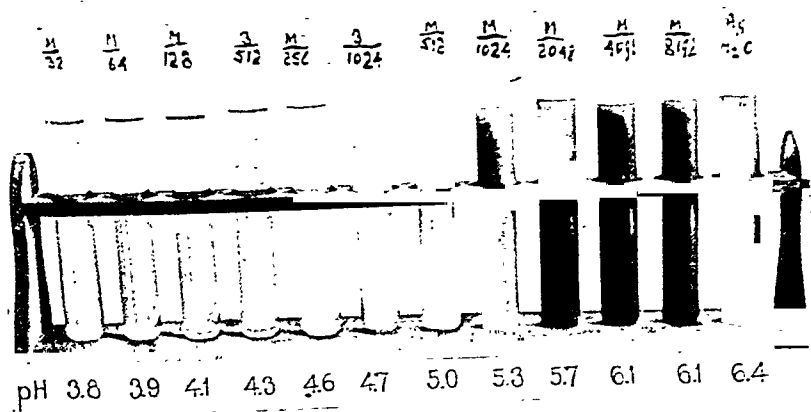| pH 3.8 | 3.9 | 4.1 | 4.3 | 4.6 | 4.7 | 5.0 | 5.3 | 5.7 | 6.1 | 6.1 | 6.4 |

FIG. 2. Photograph of the gelatin solutions whose curves are contained in Fig. 1, taken a week after the experiment was made. The gelatin with a pH > 5.0 turned dark brown in a few hours when exposed to diffused daylight, while the gelatin with a pH ⪝ 4.7 was not affected by light after an exposure of over a week. The photograph also shows that owing to the precipitate in suspension the tubes containing the gelatin at the isoelectric point, pH = 4.6 and 4.7, were more whitish than the rest, which were slightly yellow.

amount of ionic silver in combination with gelatin rises in a regular curve increasing in height with the increase in pH.

20 cc. of each gelatin solution were put into a test-tube and exposed to the light. The five test-tubes that had been treated with M/1024 or less concentrated HCl turned rapidly dark or black in the light, while none of the others did, even when made slightly alkaline and though the exposure lasted for more than a week. The gelatin treated

with 3 M/1024 acid (pH = 4.7) was quite opaque but remained perfectly white; while the test-tube treated with M/256 was only slightly opaque but remained also practically white. This shows that the gelatin treated with 3 M/1024 and whose pH was 4.7 was also entirely free from ionized silver. Fig. 2 is a photograph of these test-tubes. This experiment was repeated several times with the same result. It is a very striking demonstration experiment.

On the more acid side, where the gelatin was free from silver, the gelatin existed as gelatin nitrate as we shall prove presently. This then shows that at the isoelectric point and on the acid side from the isoelectric point a cation cannot combine with gelatin, while on the more alkaline side from the isoelectric point such a combination occurs.

Table I gives the cc. of 0.01 N Ag found in combination with 0.25 gm. of gelatin.

TABLE I.

Cc. 0.01 N Ag in combination with 0.25 gm. of gelatin at different pH.													
pH........ . ... 3.6	3.8	3.8	3.9	4.1	4.3	4.6	4 7	5.0	5.3	5.7	6.1	6 1	6 4
Cc. 0.01 N Ag combined with 0.25 gm. of gelatin.. 0.5	0.3	0.4	0.3	0.2	0.2	0.2	0.55	1.25	3.2	4 0	4.85	4.6	4 9

The small values of 0.2 cc. or even 0.5 cc. found in the gelatin on the acid side from the isoelectric point are due to incomplete removal by washing; an increase in the number of washings would probably have removed these traces also. Where 0.5 or less cc. of 0.01 N Ag was found in 0.25 gm. of gelatin, light no longer blackened the gelatin. As soon as pH became greater than 4.7 the value of Ag found rose suddenly.

Fig. 3 represents the physical properties of gelatin treated first with M/16 AgNO₃ and then brought to different hydrogen ion concentrations by treatment with different concentrations of HNO₃. 1 gm. of powdered gelatin was first put for 30 minutes into 100 cc. of M/16 AgNO₃ (pH about 6.9) and the excess of salt was then washed away (in the manner described). Then each gm. of gelatin was perfused three times with a definite concentration of HNO₃ and the excess of acid was washed away by four perfusions with distilled water. The
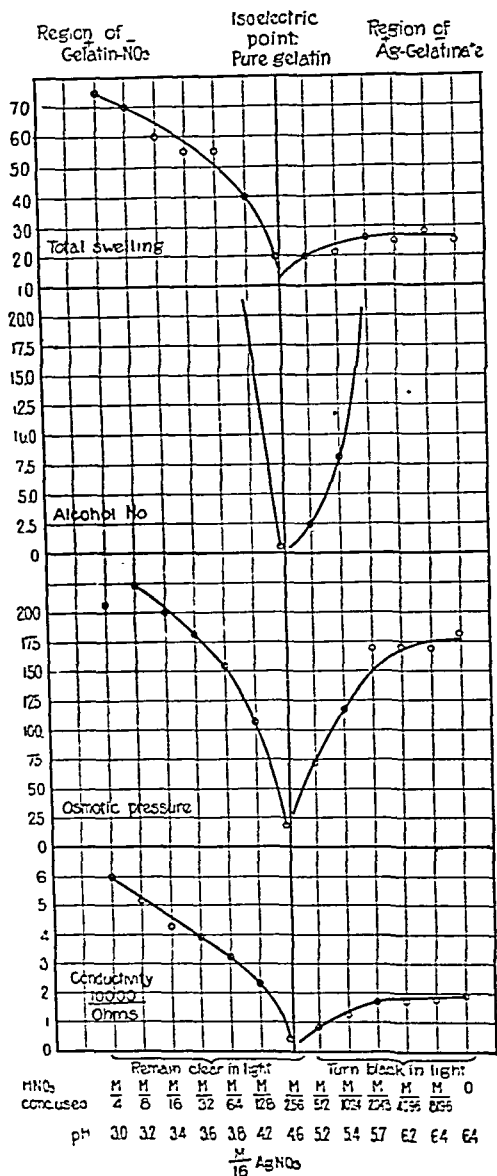
FIG. 3. Gelatin treated first with м/16 AgNO₃, washed, then treated with different concentrations of HNO₃, and washed again. Curves for conductivity, osmotic pressure, alcohol number, and swelling. On the right from the isoelectric point the gelatin exists as silver gelatinate, on the left as gelatin nitrate, and at the isoelectric point as pure, sparingly soluble gelatin.

243

swelling of the gelatin was measured while it was in the cylindrical funnel and the height of the column of gelatin in mm. expresses the degree of swelling, all the funnels having the same diameter. The gelatin was then melted, made into a 1 per cent solution, and the conductivity, osmotic pressure (in mm. of the height of the gelatin solution), alcohol number (cc. of 95 per cent alcohol required to cause a definite degree of precipitation in 5 cc. of 1 per cent gelatin solution) were determined. There is an abrupt fall in all the curves near pH 4.6; *i.e.*, at about the isoelectric point, and a rise on both sides. On the right from the isoelectric point (pH > 4.7) the gelatin exists as silver gelatinate, while on the left, more acid side from the isoelectric point (pH < 4.7) the gelatin exists as gelatin nitrate. At the isoelectric point it exists as non-ionized gelatin. This was proved by exposing the gelatin solutions to the light after completion of the experiment which was of course carried out in a dark room; all those gelatin solutions whose pH was from 5.3 to 6.4 turned black when exposed to light, while none of the others was affected by light even after an exposure of more than 2 weeks. Since the original treatment of the gelatin with $M/16$ $AgNO_3$ near the point of neutrality was the same for all the solutions, it is obvious that the silver gelatinate when its pH was 4.7 or less gave off part if not practically all its silver. This was actually proved to be the case by the analysis of the filtrate of such gelatin during and after the acid treatment.

When we treat the gelatin first with HNO_3, wash the acid away, and then treat the gelatin with $M/16$ $AgNO_3$ and wash away the excess of salt, we get an identical system of curves and an identical effect of exposure to light, proving that gelatin can only combine with Ag when its pH > 4.7, while it cannot combine with gelatin when its pH $\lessgtr$ 4.7.

The writer would like to call attention to the fact shown in Figs. 1 and 3 that for pH > 4.7 the curves for swelling and for conductivity are practically parallel to the curve for the amount of silver gelatinate formed.

The Combination of Anions with Gelatin.

In this chapter we intend to furnish the data necessary for proving that gelatin combines with anions only when pH < 4.7, while it can-

not combine or remain in combination with anions when pH $\gtrless$ 4.7. We chose for this purpose two anions, Br and CNS, for the volumetric determination of which we possess the convenient Volhard method. Powdered gelatin was first treated with different concentrations of HNO_3 and then washed free from the excess of acid. Then the gelatin in each funnel was perfused three times with 25 cc. of M/8 NaBr and this was followed by six perfusions with 25 cc. of H_2O. The swelling was plotted (upper curve in Fig. 4), the gelatin was melted and made into a 1 per cent solution, and analyzed for Br by the Volhard method. The lower curve gives the quantity of Br in combination with 25 cc. of gelatin. Table II gives the amount of Br found in combination with 0.25 gm. of gelatin. The table, as well as the curve, shows that the treatment of gelatin with M/8 NaBr did not lead to the formation of gelatin bromide when pH $\gtrless$ 4.7; while it led to the formation of gelatin Br when pH < 4.7. The quantity of gelatin bromide formed increased with the hydrogen ion concentration.

TABLE II.

Cc. 0.01 N Br in combination with 0.25 gm. of gelatin at different pH.

pH	3.7	3.8	3.9	4.1	4.2	4.6	4.7	5.1	5.8	6.4	6.8	7.0	7.1
Cc. 0.01 N Br combined with 0.25 gm. of gelatin	7.5	7.4	7.1	5.0	2.95	1.4	0.1	0.15	0.1	0.15	0.2	0.1	0.1

In the experiment represented by Fig. 5 everything was the same except that the treatment of gelatin with HNO_3 was followed by three perfusions with M/8 NH_4CNS (instead of M/8 NaBr). The lower curve shows that when pH $\gtrless$ 4.7 the gelatin contains no CNS, but that gelatin can combine with CNS as soon as pH < 4.7.

The results are given in Table III. The reader's attention is called to the sharp drop in the values for Br (Table II) as well as for CNS (Table III) at pH = 4.7 and less.

TABLE III.

Cc. 0.01 N CNS in combination with 0.25 gm. of gelatin at different pH.

pH	3.5	3.5	3.9	4.1	4.3	4.4	4.7	4.9	5.5	6.2	6.3	6.4	6.5	7.0
Cc. 0.01 N CNS combined with 0.25 gm. of gelatin	9	9	6.7	4.1	3.4	1.95	0.15	0.2	0.1	0.1	0.1	0.1	0.1	0

When we reverse the order of treatment, *i.e.* when we treat gelatin first with M/8 NaBr at pH about 7.0, only sodium gelatinate should be formed according to our theory, and no Br should combine with the gelatin.   This was found to be correct.   When we treat gelatin first
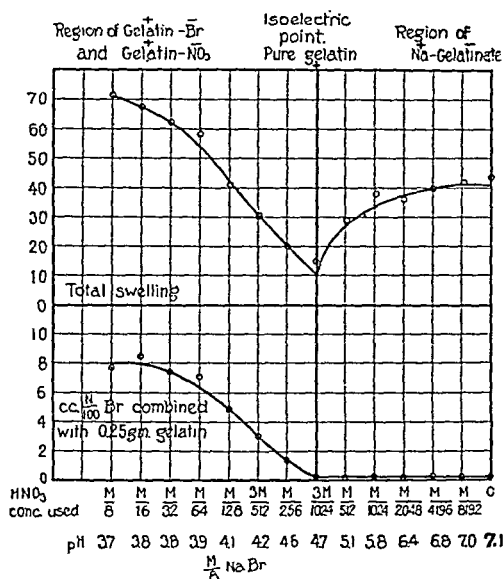

FIG. 4. Gelatin treated with different concentrations of HNO₃, washed, treated with M/8 NaBr, and washed again.   The ordinates of the lower curve are the values of Br in combination with gelatin, showing that on the right (alkaline) side from the isoelectric point and at the isoelectric point gelatin contains no Br, while on the left, more acid side from the isoelectric point, the amount of the Br found increases with the pH.   The ordinates of the upper curve represent the swelling of the same gelatin.   The two curves on the left side are almost parallel, showing that the degree of swelling is determined by the relative mass of the gelatin bromide (or nitrate) formed.

with M/8 NaBr, then with varying concentrations of HNO₃, no Br should enter into ionogenic combination with the gelatin.   This is confirmed by the volumetric analysis which shows that such gelatin is free from ionic Br.   Only sodium gelatinate is formed under these conditions.

Figs. 4 and 5 confirm a fact already referred to in connection with Fig. 1.   The curves for the swelling of gelatin for pH < 4.7 are parallel to the curves representing the amount of gelatin Br and gelatin CNS formed.   Fig. 6 gives the relation of the curve for swelling to the curves for the other physical properties of gelatin treated with acid and then with M/8 NaBr.   These experiments prove definitely that on the acid side from the isoelectric point gelatin (and probably amphoteric colloids in general) can combine only with anions, on the
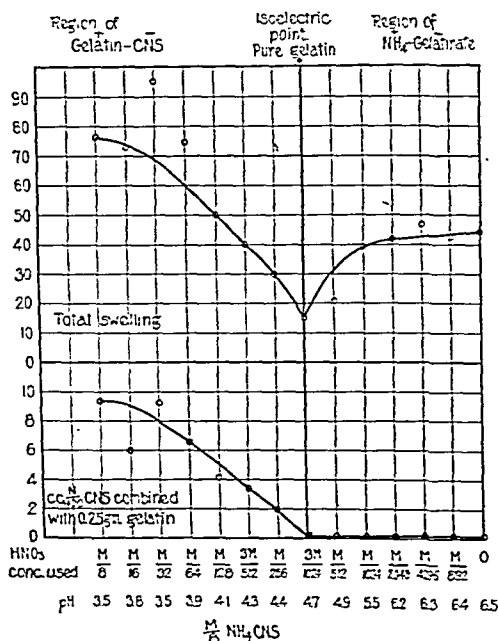
FIG. 5. Gelatin treated with different concentrations of HNO₃, washed, then treated with M/8 NH₄CNS, and washed again.   Further explanation of figure corresponds to that for Fig. 4.

more alkaline side from the isoelectric point only with cations, and at the isoelectric point with neither. .

Action of Heavy Metals and Polyvalent Ions.

When we treat finely powdered gelatin with a solution of M/10 copper acetate, the powder assumes a blue color due to the formation
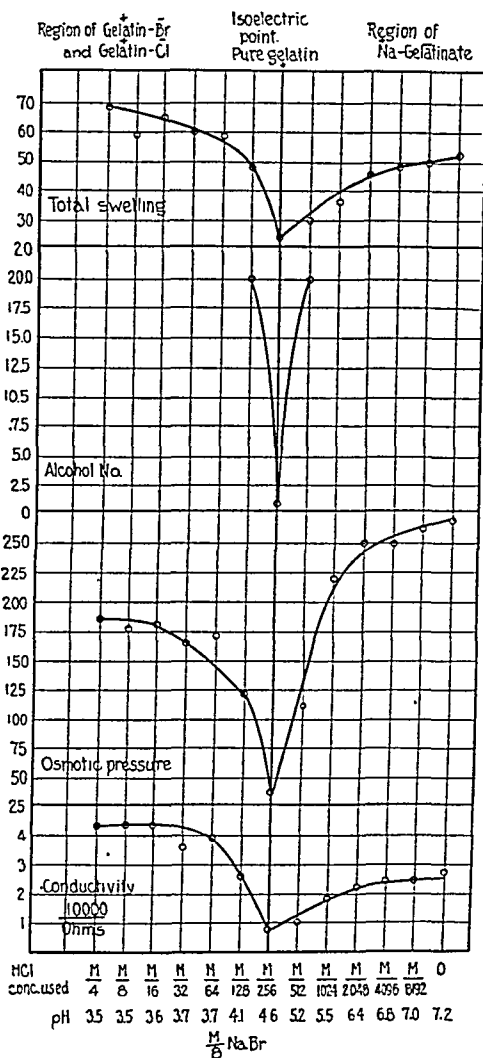
FIG. 6. Gelatin treated first with varying concentrations of HCl, washed, then treated with м/8 NaBr, and washed again.   Curves for conductivity, osmotic pressure, alcohol number, and swelling.   On the right side from the isoelectric point gelatin exists as sodium gelatinate, on the left side as gelatin chloride or bromide, at the isoelectric point as pure, sparingly soluble gelatin.

of copper gelatinate (pH being about 6.3). When we wash off the excess of copper acetate solution, the blue color remains. When we treat such copper gelatinate with an acid to increase the hydrogen ion concentration, we notice that the gelatin loses its blue color as soon as its pH $\lessgtr$ 4.7. If after the acid treatment gelatin is perfused six times with distilled water, the gelatin keeps its blue color when pH $>$ 4.7, and it is possible to demonstrate the presence of ionized copper in such gelatin with the ferrocyanide test. When the pH of the gelatin is $\lessgtr$ 4.7, the ferrocyanide test shows that the gelatin is free from ionized copper. The water with which this gelatin was washed contains the copper, while the water with which the gelatin with a pH $>$ 4.7 was washed is free from copper, except that which was originally contained in the capillary spaces between the granules of gelatin and which was carried away with the first perfusions.

Fig. 7 gives the curves of gelatin first treated for 30 minutes with $M/16$ copper acetate and subsequently with from $M/8$ to $M/8192$ HCl. For pH $\gtrless$ 4.6 all the values of the curves are as low as they are at the isoelectric point, and for the same reason; namely, copper gelatinate as well as the gelatin at the isoelectric point being sparingly soluble. (It is well to keep in mind that at the isoelectric point the gelatin contains no ionizable copper, while for pH $>$ 4.7, the gelatin is in combination with ionic copper.) On the left, more acid side from the isoelectric point the gelatin exists in the form of gelatin chloride and gelatin acetate.

The experiment proves, therefore, that copper gelatinate cannot continue to exist when pH $\lessgtr$ 4.7, and that the gelatin gives off its copper at such pH.

The same experiment was made with $CeCl_3$ and Pb acetate$_4$, and almost identical curves were obtained as in the case of copper acetate, Ce as well as Pb forming sparingly soluble gelatinates. In the case of K_4FeCN_6 the sparingly soluble gelatin$_4$ $FeCN_6$ was formed only on the acid side of the isoelectric point as our theory demands. Fig. 8 gives the curves. The gelatin was first treated with different concentrations of HCl and then with $M/16$ K_4FeCN_6 in the manner described for the other cases.
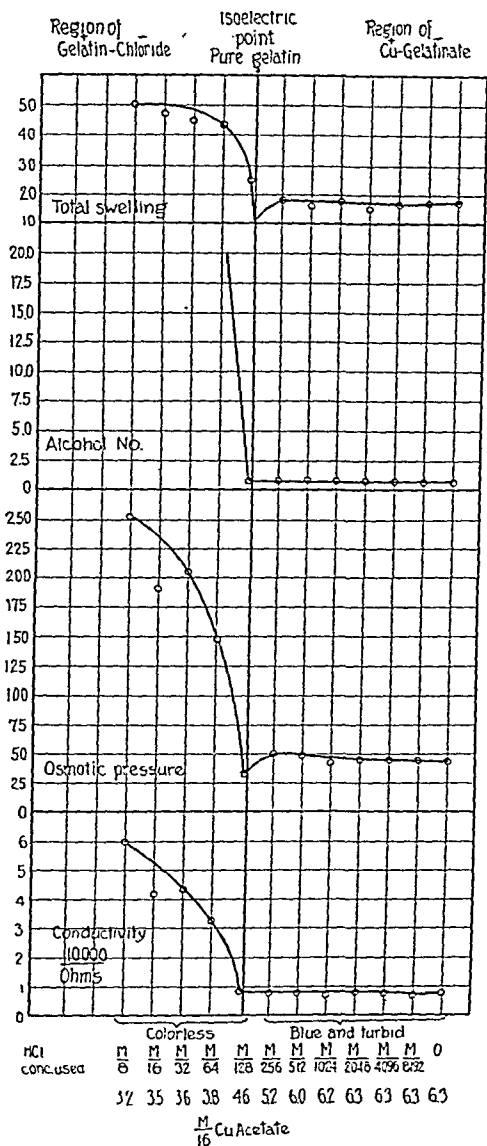
FIG. 7. Gelatin treated with m/16 copper acetate, washed, treated with different concentrations of HCl, and washed again. On the right side from the isoelectric point gelatin exists as copper gelatinate, stained blue, practically insoluble, and with low conductivity, osmotic pressure, alcohol number, and swelling. On the left side from the isoelectric point it exists as gelatin chloride (and acetate), highly soluble, and with high conductivity, osmotic pressure, alcohol number, and swelling; at the isoelectric point pure gelatin, practically insoluble.

250
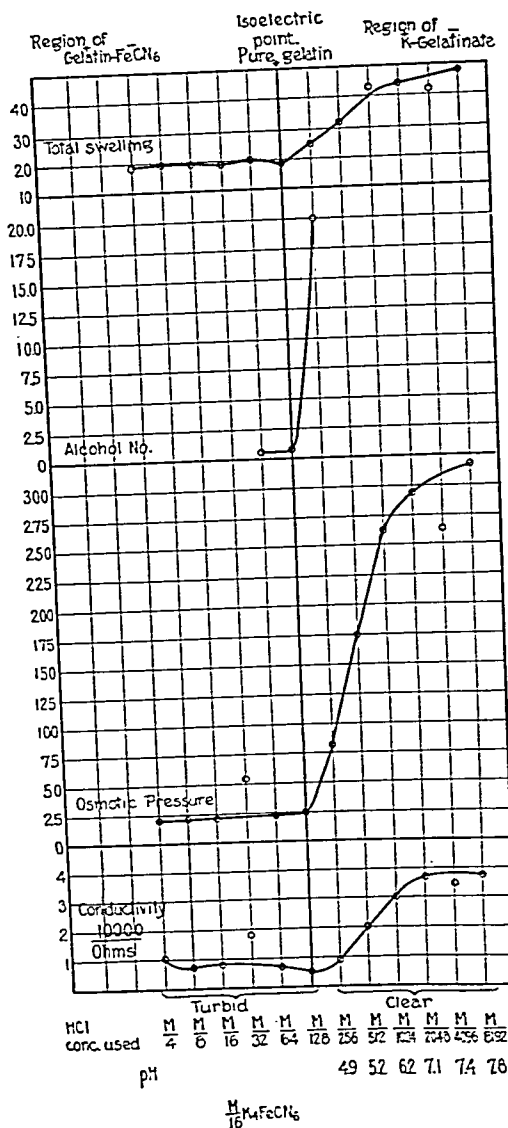
FIG. 8. Gelatin treated with different concentrations of HCl, washed, then treated with м/16 K₄FeCN₆, and then washed again. On the left, more acid side from the isoelectric point, gelatin exists as insoluble gelatin ferrocyanide, with low osmotic pressure, low conductivity, etc.; on the right, more alkaline side from the isoelectric point, gelatin exists as potassium gelatinate with high conductivity, osmotic pressure, etc.; at the isoelectric point, pure and sparingly soluble gelatin.

Experiments with Dyes.

Gelatin is readily stained with the basic dye neutral red at the point of neutrality. Our theory demands that gelatin thus stained with neutral red should give off all its stain at the isoelectric point and on the acid side from the isoelectric point; while the neutral red should be retained when pH > 4.7. This turns out as the theory demands. 1 gm. of finely powdered gelatin was put for 20 minutes at about 15°C. into a series of beakers each containing 1 cc. of M/100 neutral red. The gelatin was then filtered and washed twice with 25 cc. of distilled water. All the gelatin was stained a beautiful deep red. Each funnel was then perfused three times with a definite concentration of HCl, the concentration for the various funnels varying from M/8 to M/8192, and this was followed by eight perfusions with 25 cc. of H_2O each. In the funnels treated with a concentration of HCl of M/512 or above, the gelatin became decolorized (the neutral red being washed away); while all the funnels treated with acid of a lower concentration retained their deep red color and did not give off their stain. The decolorized gelatin was then melted, made into a 1 per cent solution whose pH was determined. It was found that the gelatin previously treated with M/512 HCl and decolorized had a pH = 4.7, that treated with 3 M/1024 having a pH = 4.6, and so on. Hence the gelatin was not able to retain its neutral red when brought to the isoelectric point or to a pH $\lessgtr$ 4.7, as our theory demands.

Michaelis and Davidsohn[3] working with large blocks of gelatin, gained the impression that gelatin is slightly stainable with both basic and acid stains at the isoelectric point. We are inclined to believe that our method of staining gelatin in a finely divided condition and then ascertaining the pH where the dye is given off again by the gelatin, gives more reliable results than their method of working with large blocks of gelatin. We intend to return to the problem of staining in a future communication.

SUMMARY.

1. It is shown by volumetric analysis that on the alkaline side from its isoelectric point gelatin combines with cations only, but not

---

[3] Michaelis. L., and Davidsohn, H., *Biochem. Z.*, 1913, liv, 323.

with anions; that on the more acid side from its isoelectric point it combines only with anions but not with cations; and that at the isoelectric point, pH = 4.7, it combines with neither anion nor cation. This confirms our statement made in a previous paper that gelatin can exist only as an anion on the alkaline side from its isoelectric point and only as a cation on the more acid side of its isoelectric point, and practically as neither anion nor cation at the isoelectric point.

2. Since at the isoelectric point gelatin (and probably amphoteric colloids generally) must give off any ion with which it was combined, the simplest method of obtaining amphoteric colloids approximately free from ionogenic impurities would seem to consist in bringing them to the hydrogen ion concentration characteristic of their isoelectric point (*i.e.*, at which they migrate neither to the cathode nor anode of an electric field).

3. It is shown by volumetric analysis that when gelatin is in combination with a monovalent ion (Ag, Br, CNS), the curve representing the amount of ion-gelatin formed is approximately parallel to the curve for swelling, osmotic pressure, and viscosity. This fact proves that the influence of ions upon these properties is determined by the chemical or stoichiometrical and not by the "colloidal" condition of gelatin.

4. The sharp drop of these curves at the isoelectric point finds its explanation in an equal drop of the water solubility of pure gelatin, which is proved by the formation of a precipitate. It is not yet possible to state whether this drop of the solubility is merely due to lack of ionization of the gelatin or also to the formation of an insoluble tautomeric or polymeric compound of gelatin at the isoelectric point.

5. On account of this sudden drop slight changes in the hydrogen ion concentration have a considerably greater chemical and physical effect in the region of the isoelectric point than at some distance from this point. This fact may be of biological significance since a number of amphoteric colloids in the body seem to have their isoelectric point inside the range of the normal variation of the hydrogen ion concentration of blood, lymph, or cell sap.

6. Our experiments show that while a slight change in the hydrogen

ion concentration increases the water solubility of gelatin near the isoelectric point, no increase in the solubility can be produced by treating gelatin at the isoelectric point with any other kind of monovalent or polyvalent ion; a fact apparently not in harmony with the adsorption theory of colloids, but in harmony with a chemical conception of proteins.

The writer wishes to express his thanks to Mr. M. Kunitz for his faithful assistance in these experiments.

MONOGRAPHS OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARC

Under the head of *Monographs of The Rockefeller Institute for Medical Research* are published f time to time scientific papers which are so extensive, or require such elaborate illustrations, a render them unsuitable for current periodical issues. *Monographs* will be sent post paid on a cation, at $1.00 each, payable in advance by check or money order.

Monograph No. 1. (Issued June 30, 1910.)|

SIMON FLEXNER and J. W. JOBLING. Studies upon a transplantable rat tumor. Plates 1 J. W. JOBLING. The biology of a mixed tumor of the rat. MAUD L. MENTEN. Exp ments on the influence of radium bromide on a carcinomatous tumor of the rat. J JOBLING. Spontaneous tumors of the mouse. Plates 17–28. J. W. JOBLING. Transp tation experiments in *Macacus rhesus* with a carcinomatous teratoma from man.

Monograph No. 2. (Issued February 15, 1911.)

WILLIAM TRAVIS HOWARD and OSCAR T. SCHULTZ. Studies in the biology of tumor c Plates 1–6.

Monograph No. 3. (Issued March 15, 1911.)

BENJAMIN T. TERRY. Chemo-therapeutic trypanosome studies with special reference to immunity following cure.

Monograph No. 4. (Issued June 24, 1912.)

FRANCIS W. PEABODY, GEORGE DRAPER, and A. R. DOCHEZ. A clinical study of poliomyelitis. Plates 1–13.

Monograph No. 5. (Issued September 27, 1915.)

J. P. SIMONDS. Studies in *Bacillus welchii*, with special reference to classification and to its tion to diarrhea.

Monograph No. 6. (Issued January 31, 1916.)

JAMES L. STODDARD and ELLIOTT C. CUTLER. Torula infection in man. Plates 1–9

Monograph No. 7. (Issued October 16, 1917.)

OSWALD T. AVERY, H. T. CHICKERING, RUFUS COLE, and A. R. DOCHEZ. A lobar pneumonia. Prevention and serum treatment. Plates 1–3.

Monograph No. 8. (Issued July 31, 1918.)

ERNEST C. DICKSON. Botulism. A clinical and experimental study. Plates 1–10.

Monograph No. 9. (In press.)

JAMES HOWARD BROWN. The use of blood agar for the study of streptococci. Plates 1–34

THE JOURNAL

OF

GENERAL PHYSIOLOGY

PUBLICATIONS OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

THE JOURNAL OF EXPERIMENTAL MEDICINE

EDITED BY

SIMON FLEXNER, M.D.

The Journal of Experimental Medicine is designed to cover the field of experimental medicine. It is issued monthly, two volumes of over 600 pages each appearing in a year. The subscription price per year is $5.00 (two volumes); single copies, 75 cents.

THE JOURNAL OF BIOLOGICAL CHEMISTRY

EDITED BY

H. D. DAKIN, New York, N. Y.

E. K. DUNHAM, New York, N. Y.

LAFAYETTE B. MENDEL,
New Haven, Conn.

A. N. RICHARDS, Philadelphia, Pa.

DONALD D. VAN SLYKE, New York, N. Y.

CLARENCE J. WEST, New York, N. Y.

The Journal of Biological Chemistry is issued monthly, and since its foundation in 1905 thirty-six volumes of approximately 550 pages each have been completed. The subscription price is $3.00 per volume to subscribers in the United States and Canada; $3.25 to those in other countries. The number of volumes issued yearly averages four.

THE JOURNAL OF GENERAL PHYSIOLOGY

EDITED BY

JACQUES LOEB, New York, N. Y.

W. J. V. OSTERHOUT, Cambridge, Mass.

The Journal of General Physiology is devoted to the explanation of life phenomena on the basis of the physical and chemical constitution of living matter. Information as to contributions and subscriptions is given in full on the back cover.

STUDIES FROM THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

The published results of investigations conducted in the Institute, or under its grants, are republished at irregular intervals in volumes designated *Studies from The Rockefeller Institute for Medical Research*. Price, $2.00 per volume, payable in advance. A special rate of $1.00 per volume is made to those who subscribe to *The Journal of Experimental Medicine, The Journal of Biological Chemistry,* or *The Journal of General Physiology.*

SEMIANNUAL LIST

The Institute publishes a *Semiannual List* of all papers by members of the staff and those working under grants from the Institute, stating the title and place of publication of these reports. The *Semiannual List* will be sent regularly upon application.

THE ANTAGONISM BETWEEN THYMUS AND PARATHYROID GLANDS.

By EDUARD UHLENHUTH.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

INTRODUCTION.

It is well known that the extirpation of the parathyroids in mammals is followed by tetanic convulsions. This led to the conclusion

CORRECTION.

The accompanying pages are to replace pages 23 and 24 of *The Journal of General Physiology*, September 20, 1918, Vol. i, No. 1. In the first line of the ... e as originally printed *thyroid* was written for *thymus*.

Ambystoma maculatum[2] were fed exclusively on calf's thymus. Each single larva after some time began to suffer from severe tetanic attacks. Since the larvæ of salamanders do not possess parathyroids this observation seemed to be of considerable interest, and, in the fall of 1916, calf's thymus was fed to larvæ of another species of sala-

[1] See Biedl, i, 279, 301 ff.

[2] In the terminology to be employed in the classification of amphibians the nomenclature as worked out by Stejneger and Barbour in their new check list was applied here. According to this the old species *Amblystoma punctatum* corresponds to the new species *Ambystoma maculatum*.

manders, *Ambystoma opacum;* again each single individual after a certain time came down with tetanic attacks. In 1917, the experiments were repeated with the same result on both species. It is, therefore, evident that the thymus gland contains a substance which produces tetanic convulsions in the larvæ of *Ambystoma maculatum* and *opacum.*

Tetanic Symptoms.

The tetanic symptoms exhibited by salamander larvæ fed with thymus closely resemble the symptoms produced by parathyroidectomy in mammals.[3] The first attacks in the larvæ are confined to the hind portions of the body. In general, this part of the body suffers more than any other during the entire tetanic period, and when the acute attacks become less and less frequent and severe it is again the hind portions of the body which alone are attacked. Within several days after the onset of acute tetany the entire muscular system exhibits severe clonic convulsions. Each single attack begins at the tail and spreads toward the head; the tremors are severest in the legs and in the muscles of the lower jaw. During each attack the mouth is thrown widely open. When the attack begins the animals are thrown on one side. Besides the clonic convulsions a tonic spasm of the entire body is observed; the legs are stretched out and the body is bent with its concavity towards the back. During the attack the animals discharge much air and the vessels of the skin become very red.

In the early stages of the disease the convulsions are induced only upon stimulation which is best effected by removing the larvæ from the water, placing them on filter paper, and pinching the legs or the tail slightly. Each attack lasts only a short time, the larvæ recovering after from 2 to 3 minutes. Later any attempt of the larvæ to swim or to snap at a piece of food suffices to induce an attack and some individuals may float for several days on one side, being rigid from tonic spasm.

8 to 14 days after the first attack the hind legs begin to show signs of a permanent tonic spasm; the legs are stretched backward and become twisted around their longitudinal axis with the inner surface pointing upward; the feet follow this movement and finally the fore legs undergo a similar change.

[3] See Biedl, i, 79 ff.

TEMPERATURE COEFFICIENT OF THE ACTION OF β-RAYS UPON THE EGG OF NEREIS.

By ALFRED C. REDFIELD and ELIZABETH M. BRIGHT.

(From the Laboratory of Physiology, Harvard Medical School, Boston, and the Marine Biological Laboratory, Woods Hole.)

Lepper[1] has shown that grafts of rat sarcoma radiated at 15°C. show very little difference in their behavior from those radiated at 37°C., but that the latter grow rather more slowly and disappear sooner than those which have been radiated in the cold. So far as we are aware no one has determined the temperature coefficient of the physiological processes, nor of the purely chemical reactions produced by rays from radioactive substances. The velocity of photochemical processes, to which these reactions are closely allied, is known to be affected very slightly by the temperature at which the processes occur. The great mass of physiological reactions shares a high temperature coefficient (2 to 3 for a change of 10°C.) with general, non-photochemical reactions.[2] Data on the temperature coefficient of photosensory stimulation in animals do not appear to have been published. Dr. Selig Hecht has recently determined that the temperature coefficient of the sensitization process in Mya is of the order of that of a photochemical reaction (personally communicated results of unpublished experiments). Brown and Heise,[3] reviewing the literature of this aspect of photosynthesis, conclude that this important photophysiological process has a temperature coefficient of the same order (1.1 to 1.2 for a change of 10°C.) as those of photochemical reactions, although the investigators who secured the data on which this conclusion is based had assigned higher values to this

[1] Lepper, E. H., *Arch. Middlesex Hospital*, 1914, xxxiii, 77.

[2] Kanitz, A., Die Biochemie in Einzeldarstellungen; Temperature und Lebensvorgänge, Berlin, 1915, pt. 1.

[3] Brown, W. H., and Heise, G. W., *Philippine J. Sc.*, C, 1917, xii, 1.

constant. Osterhout and Haas,[4] who likewise obtain a high value, 1.81, for the temperature coefficient of photosynthesis, attribute it to the fact that they are dealing with a series of catenary reactions, of which the determining member is not a true photochemical process.

The development of a method of measuring the physiological action of radiations from radium has enabled us to determine the effect of temperature upon this process with precision. This method depends upon the fact, first observed by Packard,[5] that the fertilization membrane of the egg of the marine worm, Nereis, is greatly enlarged if the eggs have been exposed to radium prior to fertilization. We have shown[6] that the extent of this change is a reliable measure of the intensity of radiation and time of exposure. Our problem has been to determine what effect the temperature at which radiation takes place has upon the velocity of the reaction under uniform intensity.

To this end a few drops of Nereis eggs were placed in the bottom of a test-tube and this was inserted into a vessel containing chopped ice and allowed to stand until it had taken on the temperature of the ice. A tube of radium emanation was then suspended above the eggs. As radiation proceeded, a few eggs were withdrawn from time to time, placed in sea water at room temperature, fertilized, and the thickness of the membranes was measured after a uniform period of time. A second lot of eggs from the same female was then treated in exactly the same way, except that during radiation the test-tube containing them was placed in a thermos flask filled with water at room temperature.

The exposure of unfertilized eggs to these temperatures, without radiation, does not affect the volume of the membrane which is formed upon fertilization, although somewhat higher temperatures may be expected to cause an enlargement of this structure.[7]

This procedure possesses the advantage that the two lots of eggs are kept at different temperatures only during the period of radiation, during which no visible change occurs in these cells. The proc-

[4] Osterhout, W. J. V., and Haas, A. R. C., J. Gen. Physiol., 1918-19, i, 295.
[5] Packard, C., J. Exp. Zool., 1915, xix, 323.
[6] Redfield, A. C., and Bright, E. M., Am. J. Physiol., 1918, xlv, 374.
[7] Just, E. E., Biol. Bull., 1915, xxviii, 1.

ess of membrane formation does not occur until after fertilization. We have found that this process is greatly affected by temperature and have taken care that it should go on at the same temperature in both lots of eggs. Consequently we have avoided the disturbing in-
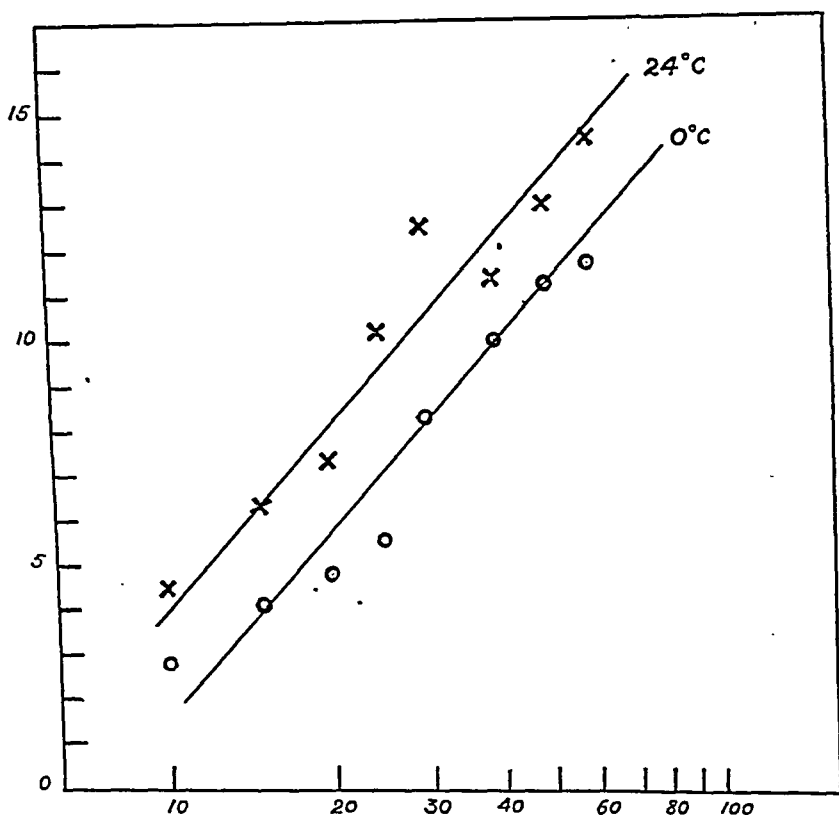
Fig. 1. Curves illustrating the effect of temperature upon the velocity of the change produced by β-rays in the membrane of Nereis eggs. Time of radiation in minutes is measured logarithmically along the abscissa. Volumes of membranes are measured in 100,000 cubic microns along the ordinate. Intensity of radiation was 38.1 millicurie centimeters in both cases.

fluence of a secondary reaction analogous to that to which the high temperature coefficient of photosynthesis is attributable.[4]

When measurements of the volumes of membranes so produced are plotted against the logarithms of the times of exposure, the points

fall about two parallel lines, as illustrated in Fig. 1. The line drawn through the points obtained at the higher temperature lies above the other, showing that the velocity of the reaction has been increased by the higher temperature. The velocities (K_1 and K_2) at the two temperatures (t_1 and t_2) are inversely proportional to the times required to produce membranes of any given volume. Thus in the experiment illustrated in Fig. 1, 40 minutes of exposure were necessary to produce a membrane of 10 hundred thousand cubic microns at 0°C. At 24°C. this membrane volume is represented by a point on the curve corresponding to an exposure of 27 minutes. We may then write $\frac{K_2}{K_1} = \frac{40}{27}$ where $t_1 = 0$ and $t_2 = 24$. Substituting these values in the equation[2]

$$Q_{10} = \left(\frac{K_2}{K_1}\right)^{\frac{10}{t_2 - t_1}}$$

we obtain 1.18 as the value of Q_{10}, the temperature coefficient for a change of 10°C. Table I gives a number of determinations of Q_{10} made in this way.

TABLE I.

t_1	t_2	Q_{10}
0	20.6	1.08
0	23.9	1.11
0	24.8	1.15
0	23.6	1.18
0	24.0	1.18
Mean		1.14

The source of radiation used in this investigation was radium emanation enclosed in slender, thin-walled glass tubes. The walls of these tubes were sufficiently thick to absorb all the α-radiation. We expect to show in a future publication that the effect of the γ-rays from such a tube is negligible compared to that of the β-rays in producing the reaction. The coefficient which we have determined is therefore that of the action of the β-rays.[8]

CONCLUSION.

The temperature coefficient of the action of β-rays from radium upon the egg of Nereis lies between 1.1 and 1.2.   This is of a magnitude characteristic of photochemical reactions.

ON THE EXPERIMENTAL PRODUCTION OF EDEMA IN LARVAL AND ADULT ANURA.

By CHARLES F. W. McCLURE.

(*From the Laboratory of Comparative Anatomy, Princeton University, Princeton.*)

Edematous frog larvæ are among the commonest types of abnormalities which can be produced by experimental means. When ova of the two- or four-celled stage are placed in solutions of potassium cyanide, acetone, butyric acid, or ethyl alcohol for from 12 to 24 hours, and then subsequently developed in tap water, an edematous condition of the body frequently, though not always, results. Hertwig[1] has produced such larvæ by exposing early cleavage stages to the influence of radium. An edematous condition of the body also occasionally results when ova are allowed to develop in direct sunlight in the laboratory and may even be met with among the controls. The life of such larvæ is relatively short.

The edematous frog larvæ thus far observed by the writer can be grouped into two main types: Type 1, in which, with the exception of their general edematous condition, the frog larvæ appear externally to be otherwise normally developed; Type 2, in which some marked abnormality, especially of the head region and vascular system, usually accompanies an edematous condition of the body.

All the larvæ referred to in this paper were in the stage at which the pronephros acts as the functional kidney and in which the mesonephros is either not at all developed, or is represented only by its earliest anlage.

An examination of serial sections of a large number of both types of edematous frog larvæ shows that in all cases, and without exception, there is a marked deficiency evident in the development of certain specified tubules in the pronephros. Reconstructions after the method of Born clearly indicate that this deficiency is associated with the

[1] Hertwig, O., *Arch. mikr. Anat.*, 2te Abt., 1911, lxxvii, 1 (see Tables II and III for examples of edematous frog larvæ).

tubules which normally constitute the greater portion of the kidney and which occupy a medial and ventral position. In some larvæ none of these particular tubules had even been developed. In all other cases, in which only their partial development had occurred, the few tubules present were always greatly hypertrophied and were often lined by an exceedingly thin-layered epithelium which closely resembled endothelium in appearance.

The less extensive tubular complex which normally occupies a dorsolateral position in the pronephros and into which the nephrostomal canals directly open was, however, invariably found to be developed to a considerable degree in all the edematous larvæ examined. It was lined by a thick-layered epithelium which is the only epithelium of the pronephros in the writer's experience, in both normal and edematous frog or toad larvæ, that has thus far been observed to absorb colloidal acid dyes. In all cases this dorsolateral tubular complex was found to be much hypertrophied and often bladder-like in appearance and, together with the three nephrostomal canals which open into it, was occasionally found to constitute the only portion of the kidney that had been formed. In such extreme cases the pronephric duct was either a solid structure or was not connected with the kidney at all.

Associated with this general defect in kidney development the lymph sinuses of the body, especially those of the head region, were often found to be greatly distended with fluid. In larvæ of Type 1 in which the edema is generalized, no defect in the development of any organs of the body was evident except that of the pronephric tubules referred to. In some cases (Type 2) the anterior lymph hearts were absent, and other prominent defects in the vascular system met with which need not be mentioned here. The pericardial and cœlomic cavities were often greatly expanded and blebs or blisters of various sizes were occasionally formed under the epidermis. All these cavities were filled with fluid, as could easily be determined in larvæ which had been placed in solutions of colloidal acid dyes.

We know that water and certain colloidal acid dyes[2] are continu-

[2] McClure, C. F. W., On the behavior of *Bufo* and *Rana* toward colloidal dyes of the acid azo group, Memoirs of The Wistar Institute of Anatomy and Biology, No. 8, Philadelphia, 1918.

ously absorbed through the integument in both larval and adult frogs and toads when the latter are allowed to remain in such media, and that the excess of fluid taken into the body in these cases is normally eliminated by the kidneys. Since the water taken into the body by absorption through the integument must necessarily reach the kidneys by way of the vascular system, the possibility suggests itself that the edematous condition of the frog larvæ referred to may be due to the deficiency observed in the development of certain specified tubules of the kidney, or, in other words, to a block in the normal kidney function. In consequence of this an excess of water which, is constantly being taken into the body through the integument is retained in the body, since it can no longer be eliminated by the kidneys. That this is actually the case, as far as these particular frog larvæ are concerned, seems to be borne out by experiments on adult frogs and toads in which an edematous condition of the body can be produced by ligation of the ureters.

The distal ends of the ureters in the frog and toad lie in the dorsal wall of the cloaca and open dorsally into the latter-independently of the bladder, which opens on the ventral wall of the cloaca. It is therefore not a difficult matter to ligate the ureters by means of artery forceps and small metal clamps, or to pass a ligature around the ureters which can be tied over the urostyle. In such cases neither the bladder nor the outlet of the intestinal canal through the cloaca is interfered with.

Among 53 ligation experiments thus far made by the writer, chiefly on adult toads, the six mentioned in Table I illustrate the range of results obtained. In all cases the toads[3] were kept in water for a considerable time and weighed at intervals before applying the ligature. This was done in order to establish a normal balance, under such conditions, between the absorption of water through the integument and its elimination from the body by the kidneys.

The first figure in each of the columns of Table I (Experiments 1 to 6) indicates the weight of the toad in gm. at the time the ureters were ligated and before the toad was again placed in water. The succeeding figures in each column indicate the observations made at different intervals of time.

[3] *Bufo lentiginosus* Shaw.

TABLE I.

Time.	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5	Experiment 6
hrs.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
0	22.8	30.5	30.9	45.9	53.9	49.1
0.50		32.7				
0.52				51.5		
1.10					54.5	
1.35		34.2				
1.40	23.75			54.5		
2.20				57.0	55.4	
2.43	24.8					
3.35			34.0			
5.05	26.9					
5.45				64.4 (40%)		
6.10	27.6					
6.55						55.9
7.45	28.7					
11.55	31.2					
16.55		50.8 (66.5%)				
17.55					60.3	
19.00					60.9	
20.00					61.7	
20.50					62.7	
23.25			58.6 (89.6%) Removed ligature.			
23.30					68.4	
23.40	37.5 (64.4%)					
24.05					70.5 (30%)	
25.25			47.5			52.2
25.55			43.9			
28.35			37.9			56.1
29.55			35.2			
30.50			32.9			
31.55			30.9			
48.40						68.4 (39.3%)

These experiments should not be confused with the well known experiments of Overton[4] who ligated the cloacal opening but not the ureters. When only the cloacal opening is ligated, the flow of urine through the ureters into the cloaca and bladder is not interrupted. The urine in such cases, as stated by Overton, will fill up the bladder and then back up into the intestinal canal. When the ligature is removed, this urine can be readily expressed from the body through the cloaca. On the other hand, as we shall see in the following experiments, after removal of the ligature from the ureters, no fluid can ordinarily be immediately expressed from the cloaca as, in this case, the excess of fluid contained in the body has been deposited in the tissues and body cavity, and not in the bladder and intestinal canal. The six experiments referred to in Table I were made during the month of August.

Experiment 1.—When killed the toad was in a comatose condition and had gained 64.4 per cent in weight in 23 hours and 40 minutes. On removal of the silk ligature no fluid was passed from the cloaca. A clear fluid containing albumin and weighing 6.4 gm. was removed from the subcutaneous lymph sinuses. A clear fluid weighing 4.9 gm. which threw down a precipitate in nitric acid that disappeared when acetic acid was added, was also removed from the body cavity. An excess of fluid amounting to 3.4 gm. still remained in the tissues after removal of the fluid from the lymph sinuses and body cavity. The lungs were inflated and intensely congested. The blood vessels, especially those of the abdomen, integument, and kidneys were also greatly congested.

Experiment 2.—When killed the toad was partially comatose and had gained 66.5 per cent in weight in 16 hours and 55 minutes. No fluid could be expressed from the cloaca on removal of the small metal clamp with which the ureters had been ligated. A bloody fluid weighing 11.6 gm. was removed from the subcutaneous lymph sinuses and 3.2 gm. of fluid of a similar character were found in the body cavity, leaving an excess of fluid amounting to 5.5 gm. in the tissues of the body after removal of fluid from the lymph sinuses and body cavity. The condition of the lungs and blood vessels was essentially the same as in Experiment 1.

Experiment 3.—In this case the dorsal wall of the cloaca was tightly clamped with an artery forceps, the teeth of which undoubtedly cut deeply into the tissues. After being placed in water the toad gained 89.6 per cent in weight in

---

[4] Overton, E., *Verhandl. physik.-med. Ges. Würzburg*, 1904, xxxvi.

the course of 23 hours and 25 minutes, at the end of which time the forceps was removed. The toad was in a comatose condition and a few drops of fluid could be expressed from the cloaca. The toad was not killed, and at the end of 8 hours and 30 minutes, after removal of forceps, had regained its normal weight and was very active.

Experiment 4.—When killed the toad had gained 40 per cent in weight in the course of 5 hours and 45 minutes and was still quite active. In ligating the ureters with a silk ligature which passed under the ureters and over the urostyle, a large blood vessel was punctured. The subcutaneous lymph sinuses of the body contained 7.1 gm. of a clear fluid which coagulated on exposure to air, and the body cavity 4.3 gm. of a fluid in which considerable blood was present. The excess of fluid remaining in the tissues after removal of fluid from the lymph sinuses and body cavity amounted to 7.1 gm. The lungs, kidneys, and subcutaneous blood vessels were intensely congested. .

Experiment 5.—In this instance the ureters were compressed by a small metal clamp, the jaws of which pressed tightly upon, but did not cut into the tissues. When the toad was killed it was very weak and had gained only 30 per cent in weight in the course of 24 hours and 5 minutes. On removal of clamp no fluid could be expressed from the cloaca. About 7.7 gm. of a slightly bloody fluid were removed from the subcutaneous lymph sinuses and only 1 gm. of a clear fluid containing albumin was found in the body cavity. The excess of fluid still remaining in the tissues after its removal from the lymph sinuses and body cavity amounted to 7.9 gm. The lungs were greatly expanded and intensely congested. Marked signs of congestion were observed on the abdomen, especially in the region of the pelvis.

Experiment 6.—This illustrates an instance in which a gradual increase in body weight is followed by a slight decrease, and then succeeded by a gradual and progressive increase in body weight which develops into an intense form of generalized edema. In this particular case an artery forceps was used in which the teeth had been filed down so that they would not cut into the tissues, and the pressure exerted on the ureters was also relatively slight. When the toad was killed it had gained only 39.7 per cent in weight in the course of 48 hours and 40 minutes. The slow progress of the edema was probably due to an incomplete ligation of the ureters. On removal of forceps about 2.1 gm. of semifluid excreta were expressed from the cloaca. The fluid removed from the subcutaneous lymph sinuses was clear, coagulated on exposure to air, and amounted to 4.2 gm. The fluid present in the body cavity was extremely bloody and also weighed 4.2 gm. The excess of fluid still remaining in the tissues after its removal from the lymph sinuses and body cavity amounted to 8.8 gm. The lungs, kidneys, left oviduct, mesenteries, and subcutaneous blood vessels were intensely congested.

The toad was weighed twenty-one times in the course of this experiment. Only the more critical data, however, are mentioned in Table I.

The experiments clearly show when the ureters are completely ligated and the toad is placed in water, that an increase in body weight immediately takes place which gradually progresses into an intense form of generalized edema. An analysis of the conditions described need not be given here, further than that the progress of the edema appears to be related to the extent and character of the abrasion produced in the tissues at the point of ligation, as well as upon the state of congestion of the tissues which results from ligation.

On the basis of these observations it does not appear difficult to explain the edematous condition of the frog larvæ mentioned above.

We know that the glomeruli of the pronephros are not directly connected with the renal tubules, as is the case in the meso- and metanephros, but project into the body cavity independently of these tubules in the vicinity of the nephrostomes. The filtrate of the pronephric glomeruli must necessarily, therefore, pass into the body cavity before entering the pronephric tubules by way of the nephrostomes. If, on account of a deficiency in their development, the tubules of the pronephros should be unable to take care of all the glomerular filtrate, or to secrete fluid obtained from the pronephric veins, an excess of fluid which could not be eliminated by the kidneys would necessarily remain in the body cavity and tissues and, in the course of time, would increase in amount so that a typical edematous condition of the body would finally result. Such I believe to be an explanation of the edematous condition observed in these frog larvæ.

Further significance of the above observations lies in the fact that they do not appear to bear out Fischer's[5] view that the cause of edema, in the anura at least, resides in the tissues, and is due to an overproduction or accumulation of acids within the body which causes them to take up water. It has also been pointed out by various authors[6] that this view is untenable on account of the limited variation of the hydrogen ion concentration in the body.

[5] Fischer, M. H., Œdema and nephritis, New York, 2nd edition, 1915.
[6] Henderson, L. J., and Cohn, E. J., *J. Am. Chem. Soc.*, 1918, xl, 857. Crozier, W. J., *J. Biol. Chem.*, 1916, xxiv, 255, 443; 1918, xxxv, 455.

· STUDIES ON BIOLUMINESCENCE.

IX. CHEMICAL NATURE OF CYPRIDINA LUCIFERIN AND CYPRIDINA LUCIFERASE.*

BY E. NEWTON HARVEY.

(From the Department of Marine Biology, Carnegie Institution of Washington, Washington, and the Physiological Laboratory, Princeton University, Princeton.)

INTRODUCTION.

In the fourth paper of this series[1] I have described the production of light by the ostracod crustacean, *Cypridina hilgendorfii,* and some of the properties of the photogenic substances found in this animal. Since then I have obtained a large number of *Cypridinæ* and have been able to carry out investigation along chemical lines on a far larger scale. This paper deals with the characteristics of two of the photogenic substances, luciferin and luciferase, in more detail. In the seventh paper[2] reasons are given for the adoption of Dubois' terms, luciferin and luciferase, which in part correspond to my photophelein and photogenin, and I have described how the oxidation product of luciferin, which I have called oxyluciferin, can be reduced to luciferin again. The chemical change involved in the oxidation of luciferin to oxyluciferin appears to be slight and comparable to the change occurring when the leuco-base of a dye is oxidized to the dye itself. It is therefore not surprising to find that oxyluciferin and luciferin have similar properties, and, as far as I have been able to

---

[1] Harvey, E. N., *Am. J. Physiol.*, 1917, xlii, 318.

[2] Harvey, *J. Gen. Physiol.*, 1918, i, 133.

determine, the properties of oxyluciferin and luciferin are identical, so that what is here recorded regarding luciferin will apply to oxyluciferin also. Unless otherwise specified, luciferin, oxyluciferin, and luciferase refer to these substances obtained from *Cypridina hilgendorfii*.

Method.

The living animals are dried quickly in desiccators over CaCl₂ and may then be kept indefinitely in well stoppered bottles containing a few lumps of $CaCl_2$ to remove any dampness in the air contained in the bottle. The *Cypridinæ* are well ground to a powder which lights brilliantly if moistened with water, both of the photogenic substances going into solution. It serves as the raw material for the isolation of luciferin and luciferase. From this material an extract may be prepared with distilled water which contains luciferin, luciferase, all the proteins of the animal soluble in water, salts, and other water-soluble material. The extract, filtered through filter paper, is yellow colored and slightly opalescent and glows for some time. The light finally disappears due to the oxidation of the luciferin. There is no change of color on standing. If the extract solution is not too concentrated and well shaken with air, all the luciferin will be oxidized and luciferase alone of the photogenic substances together with oxyluciferin will remain. Salts and other crystalloidal substances may be removed by dialysis since luciferase does not dialyze. The solution is remarkably stable. I have allowed it to dialyze against running tap water in a Schleicher and Schüll parchment diffusion thimble for 2 weeks and then against distilled water for 1 more week without any marked loss of luciferase. Indeed, solutions of luciferase may stand until they become foul and ill smelling from bacterial decomposition without destruction of the luciferase. Solution may be preserved free of bacterial development with toluene or chloroform for many months, but a slow destruction of the luciferase occurs and at the same time a precipitate forms. The luciferase is present in the colloidal state as it does not pass collodion or parchment paper membranes. A solution prepared in the above manner will be known as crude luciferase solution. Although the luciferase can be purified by repeated precipitation with a va-

riety of substances, it loses strength during the process and the crude luciferase solution is the most powerful that can be obtained.

A similar extract may be prepared with *hot* distilled water which contains one of the photogenic substances, luciferin, all of the proteins of the animal not coagulated by heating, the remaining salts, and other material soluble in hot water. My procedure is to add boiling water directly to the dry powder, boil about 20 seconds, and filter quickly while hot. The filtrate is slightly opalescent, yellow colored, and does not darken on standing. On cooling, this solution often glows faintly but if heated to the boiling point a second time the glow ceases and does not return on cooling. However, its content of luciferin is diminished because some has oxidized to oxyluciferin. This hot water extract of *Cypridinæ* will be spoken of as crude luciferin solution. It gives a bright light with luciferase.

If the hot water extract stands in a shallow dish at room temperature, the luciferin disappears completely in the course of some hours, the time depending on its concentration. This is due to oxidation of the luciferin apart from luciferase, as may be easily determined by keeping the luciferin extract in absence of oxygen. I have kept such a solution for 90 days in a test-tube covered with a layer of vaseline 1 inch in depth to prevent access of air, and at the end of that time it was capable of giving a brilliant light when mixed with luciferase. Luciferin and luciferase *together* in solution are likewise both stable in the absence of oxygen. I have kept such solutions in an evacuated tube or in a hydrogen atmosphere for many months and at the end of that time on admitting air a brilliant light appears.

The oxidative disappearance of luciferin, like other chemical reactions, is greatly accelerated at the higher temperatures. In a previous paper[1] I called attention to the fact that whereas luciferin was not destroyed by a short boiling, 3 or 4 minutes boiling were sufficient to destroy it. This is entirely a matter of the increased rate of oxidation at the boiling point. We can boil a solution of luciferin without harm for 30 minutes provided the boiling is carried out in a stream of hydrogen which has been rendered free of oxygen by passage over a glowing platinum spiral. Acid retards and alkali favors the oxidation of the luciferin (apart from luciferase). A solution of luciferin made slightly acid with HCl may be boiled for 25 minutes in the air

without complete oxidation, whereas a neutral or slightly alkaline solution is quickly oxidized.   A solution of luciferin will keep very well in a tall test-tube if the tube is left undisturbed.   Diffusion of oxygen into the depths of the tube is very slow.   Perhaps the best way to obtain a concentrated solution of luciferin is to filter the hot water extract of *Cypridina* directly into a tall narrow vessel and pass a current of CO_2 through it while cooling.   The slight acidity and the anaerobic conditions both prevent oxidation.   A little dilute acetic acid may be used in place of carbon dioxide.

Although both luciferin and oxyluciferin will pass collodion or parchment membranes, I have been unable to obtain them in crystalline form, and presume that they also are present in the colloidal state.[3] The properties of the luciferin in the hot water extract and the luciferase in the cold water extract will be considered under the following heads: Action of enzymes, salting out, alcohol and acetone, solubility in organic solvents, alkaloidal reagents, heavy metal salts, acids and alkalies, adsorbents.

Action of Enzymes.

Table I gives the results of enzyme experiments.   A solution of crude luciferase (or crude luciferin) was mixed with the enzyme preparation and kept at 38°C. in an incubator for from 18 hours to 4 days.   Controls were always employed, using previously boiled enzyme solution.   Experiments were also made to determine whether the particular enzyme preparation was active on its substrate, and no experiments were considered in which this was found not to be the case.   After the enzyme solution had acted for the proper length of time on the photogenic substances, their light-giving power was tested ·by adding an equal amount of luciferin (or luciferase) to both control and active tube and comparing the brightness of the light resulting from the active tube with that of the control.   In order to prevent oxidation the digests of luciferin were carried out in long test-tubes, full of solution, which were either corked or covered with a

---

[3] Dubois regards *Pholas* luciferin as a natural albumin and *Pholas* luciferase as an oxidizing enzyme made up of iron associated with a protein.   Dubois, R., La vie et la lumière, Paris, 1914.

thick layer of vaseline.   This procedure is not necessary in the case of luciferase.

The salivary diastase was fresh filtered saliva, and the yeast invertase a fresh filtered extract of yeast ground with sand.   All the other preparations were made by dissolving the commercial enzyme powder in water.   The erepsin was a solution of duodenal scrapings dried *in vacuo* and powdered, and the spleen, liver, and kidney substance was a solution of these glands dried quickly *in vacuo* and powdered; all were supplied by the Digestive Ferments Co. of Detroit. The three latter preparations probably contained proteolytic enzymes although they did not digest fibrin under the same conditions with which they were tested with the photogenic substances.   The erepsin in neutral solution did not digest fibrin to any extent, or albumen, but tryptophane was produced from Witte's peptone.

Since both acid and alkali injure luciferase and alkali causes a very rapid oxidation of luciferin, some difficulty was experienced in working with pepsin, active only in acid, and with trypsin, most active in alkaline solution.   As preparations containing trypsin were found to digest fibrin fairly rapidly in neutral solution, they were made up in water and the experiments show that neutral trypsin solutions will digest luciferase.   Presumably such neutral solutions would also digest luciferin if it were capable of digestion, but the results indicate that it is not.   Pepsin could only be tested on luciferase with an amount of HCl lower than the optimum, otherwise the HCl alone is sufficient to destroy the luciferase.   For this reason the action of pepsin was not so carefully investigated, but the result of the one experiment recorded indicates that a slow digestion of luciferase occurs.   Acid is not so destructive to luciferin, and 0.2 per cent HCl plus pepsin was found to possess no digestive power.

It will be noticed from Table I that of all the enzymes tried on luciferase only the proteolytic enzymes have any digestive power. Trypsin, erepsin, and pepsin HCl all have at least some digestive action.   The commercial preparations of pancreas (pancreatin) usually contain some lipase (steapsin) and diastase (amylopsin), but as salivary diastase (ptyalin) and malt diastase did not digest luciferase and a sample of trypsin lacking lipase did digest luciferase, the destructive power of various pancreatin and steapsin preparations is

Enzyme solution.	Digestive power tested.	Preservative.	Action of enzymes on luciferase, at 38°C.	
			Light with	
			24 hrs.	48 hrs.
Malt diastase*..............	Starch digestion.	None.	Brilliant.	
Same boiled...............	No digestion.	"	"	
Salivary diastase.............	Starch digestion.	None.	Brilliant.	
Same boiled...............	No digestion.	"	"	
Yeast invertase..............	Cane sugar digestion.	Toluene.		
Same boiled.............	" " "	"		
Pepsin† in less than 0.2 per cent HCl.....................	Coagulated albumen digestion.	Xylene.	Good.	
Same boiled..............	No digestion.	"	Brilliant.	
Trypsin‡.....................	Fibrin but not olive oil digestion.	Toluene.	Faint.	Negative.
Same boiled..............	No digestion.	"	Brilliant.	Bright.
Pancreatin§ in 0.5 per cent Na₂CO₃.....................	Albumen and starch digestion.	Xylene.	Fair.	Faint.
Same boiled..............	No digestion.	"	Brilliant.	Brilliant.
Pancreatin*.................	Fibrin and starch digestion.	Toluene.	Negative.	
Same boiled..............	No digestion.	"	Bright.	
Steapsin‖....................	Fat, fibrin, and starch digestion.	Toluene.	Faint.	Negative.
Same boiled..............	No digestion.	"	Brilliant.	Bright.
Rennin¶....................	Coagulated milk.	None.	Bright.	
Same boiled..............	No coagulation.	"	"	
Erepsin‖....................	Starch and peptone but not fibrin or albumen digestion.	Toluene.	Fair.	Negative.
Same boiled..............	No digestion.	"	Bright.	Bright.
Arlco urease.................	Urea hydrolyzed.	Toluene.	Bright.	
Same boiled..............	No action.	"	"	
Spleen (calf) substance‖.......	Olive oil but not fibrin digestion.	Toluene.	Good.	Faint.
Same boiled..............	No digestion.	"	Bright.	Bright.
Kidney (beef) substance‖......	Fibrin or oil not digested.	Toluene.	Good.	Fair.
Same boiled..............	No digestion.	"	"	"
Liver (hog) substance‖........	Olive oil but not fibrin digestion.	Toluene.	Good.	Faint.
Same boiled..............	No digestion.	"	Bright.	Bright.

* Merck.   † Merck, U. S. P.   ‡ Fairchild, Bros. and Foster.

| after | | | Action of enzymes on luciferin at 38°C. kept in absence of oxygen | | | | |
| rs. | 96 hrs. | Remarks. | Preservative. | Light with luciferase after | | | Remarks. |
				18 hrs.	24 hrs.	48 hrs.	
			None. "	Good. "			
ht.			None. "			Good. "	Repeated with same result.
		Repeated twice with same result.	None. "		Good. Fair.		
od.				Fair. "			Solution neutralized before testing. 0.2 per cent HCl used.
iant.			Fair. "				
	Faint. Brilliant.	Luciferase concentrated. Repeated twice with neutral pancreatin and same result.	Toluene. " .			Bright. "	Neutral pancreatin used.
			Toluene. "			Bright. "	
		Note that this solution contains trypsin.	Toluene. "			Bright. "	Repeated twice; same result.
			None. "	Fair. "			
		Repeated with same result.	Toluene. "			Bright. "	Repeated twice; same result.
at.	Negative. Bright.		None. "	Good. "			
	Fair. "		None. "		Good. "		
	Negative.		None. "		Good. "		
	Bright.		None. "		Good. "		

Davis Co.   Digestive Ferments Co.   Chr. Hansen.

unquestionably due to their trypsin content. That the destruction of luciferase is actually due to digestion and not to the injurious action of amino-acids resulting from the digestion of proteins associated. with the luciferase is shown by adding the products of a 4 day (at 38°C.) tryptic digest of albumen (then boiled to destroy the trypsin) to luciferase, and keeping the mixture with toluene at 38°C. for 4 days more. The luciferase was found to be unaffected by the amino-acids present.

These experiments all indicate that luciferase is a protein. As erepsin has a digestive action one might suppose that it belongs to the group of proteoses, but too great reliance cannot be placed on conclusions drawn from the action of erepsin, as this enzyme is said to hydrolyze histones, protamines, casein, fibrin, and nucleic acid in addition to proteoses and peptones.[4] Dubois finds that *Pholas* luciferase is digested by trypsin.[5]

On the other hand, none of the enzyme preparations tested had any action on luciferin. The proteolytic enzymes especially were studied with great care and with the result that no digestive action could be demonstrated. In one experiment not recorded in Table I luciferin was mixed with a Parke Davis Co. pancreatin preparation, having active proteolytic power and kept at 38° for 4 days without digesting the luciferin. Erepsin also had no digestive action after 4 days at 38°. Merck's pancreatin (without toluene) had no digestive action after 8 days at 38°C. Dried powdered *Cypridinæ* were mixed with Merck's pancreatin and toluene and kept at 38°C. for 8 days. This digest was found to contain no luciferase but abundant luciferin. There is no doubt of the non-digestibility of the latter. As already mentioned, these digests must be carried out in absence of oxygen, otherwise the luciferin undergoes spontaneous oxidation to oxyluciferin and disappears apart from any enzyme action. The oxyluciferin like luciferin itself is also undigested after 4 days action of a pancreatin solution at 38°C. As all common proteins, except the racemized proteins and certain very insoluble albuminoides (elastin

[4] Samuely, F., in Oppenheimer, C., Handb. Biochem. des Menschen und der Tiere, Jena, 1909, i, 554.

[5] Dubois, R., *Ann. Soc. Linn. Lyons*, 1914, xli, 161.

and keratin), are digested by trypsin, these experiments indicate that luciferin is not a common protein, but they give us no idea of the class to which it belongs.

Salting Out.

If crude luciferase solution is saturated with crystals of NaCl at 20°C. no precipitate forms but only a slight turbidity appears. The solution filters turbid and luciferase is found unharmed in the filtrate. Saturation with NaCl is a good method of preserving luciferase from the growth of moulds and bacteria.

One-half saturation with $MgSO_4$ also produces a slight turbidity and luciferase is found unharmed in the filtrate. Complete saturation with $MgSO_4$ produces a fine precipitate, again dissolving in water, which contains considerable luciferase. The opalescent filtrate also contains some luciferase so that saturated $MgSO_4$ precipitates luciferase partially but not completely.

One-half saturation with $(NH_4)_2SO_4$ produces a precipitate which contains very little luciferase after washing with half saturated $(NH_4)_2SO_4$. Most of the luciferase is found in the filtrate. On saturation with $(NH_4)_2SO_4$ an abundant precipitate forms and *no* luciferase remains in the filtrate. The $(NH_4)_2SO_4$ precipitate dissolves completely in water and gives a brilliant light if mixed with luciferin.

If a crude solution of luciferin is saturated with NaCl or half saturated with $MgSO_4$ or half saturated with $(NH_4)_2SO_4$ no precipitate forms. The luciferin remains in solution. With saturated $MgSO_4$ the luciferin is partially precipitated; with saturated $(NH_4)_2SO_4$ it is almost completely precipitated, but a small amount of luciferin still remains in the filtrate. The addition of acetic acid to the point where precipitation of the crude luciferin solution is complete (probably a nucleoprotein or mucin is precipitated) and subsequent careful saturation with $MgSO_4$ or $(NH_4)_2SO_4$ does not completely precipitate the luciferin.

The above results can be confirmed by adding saturated solutions of NaCl or $MgSO_4$ or $(NH_4)_2SO_4$ to dry powdered *Cypridinæ*. The powder glows strongly in saturated NaCl which dissolves both lucif-

TABLE II. ·

Properties of Photogenic Substances.

Property.		Luciferase.	Luciferin.
Salting out by	Saturation NaCl..................	Not precipitated.	Not precipitated.
	Half saturation MgSO₄............	" "	" "
	Saturation MgSO₄................	Nearly completely precipitated.	Partially precipitated.
	" " + acetic acid....		" "
	Half saturation (NH₄)₂SO₄........	Slightly precipitated.	Not precipitated.
	Saturation (NH₄)₂SO₄............	Completely precipitated.	Nearly completely precipitated.
	" " + acetic acid....		" " "
Solubility in	Methyl alcohol.................	Insoluble.	Soluble.
	Ethyl "	"	"
	" " 90 per cent........	"	"
	" " 70 " "	"	"
	" " 50 " "	Slightly soluble.	"
	Propyl "	Insoluble.	"
	Isobutyl "	"	Fairly soluble.
	Amyl "	"	Slightly "
	Benzyl "	"	Soluble.
	Acetone......................	"	Fairly soluble.
	" 90 per cent............	"	Soluble.
	" 70 " "	Slightly soluble.	"
	" 50 " "	Fairly soluble.	"
	Ethyl acetate.................	Insoluble.	"
	" propionate..............	"	Fairly soluble.
	" butyrate................	"	" "
	" valerate................	"	Slightly "
	" nitrate.................	"	Very slightly soluble.
	Glycerol.....................	"	Soluble.
	Glycol......................	"	"
	Ether.......................	"	Insoluble.
	Chloroform...................	"	"
	Carbon disulfide..............	"	"
	" tetrachloride...........	"	"
	Benzene.....................	"	"
	Toluene.....................	"	"
	Xylene	"	"
	Petroleum ether..............	"	"
	Aniline	"	"
	Glacial acetic acid............	"	Fairly soluble.

erin and luciferase; weakly, in saturated $MgSO_4$ which dissolves some luciferin but very little luciferase; and not at all in saturated $(NH_4)_2SO_4$ which dissolves no luciferase. On filtering the saturated $(NH_4)_2SO_4$ extract of dry *Cypridina* and pouring this into water, no light appears, but if luciferase is now added, a faint light appears, showing that a small amount of luciferin has gone into solution. These results are summarized in Table II.

Although we ordinarily think of the proteins as the substances, *par excellence*, capable of being salted out of solution, the property is characteristic of many emulsion colloids, notably soaps, polysaccharides, and phospholipins. However, neither luciferase nor luciferin is a soap because they are not precipitated by $CaCl_2$; nor phospho- or galactolipins, because they are both insoluble in ether and benzene, hot or cold. It is possible that they are of polysaccharide nature as starch and glycogen are nearly if not completely precipitated by saturating their solution with $(NH_4)_2SO_4$. But the polysaccharides are not precipitated by phosphotungstic acid, whereas luciferase is completely precipitated and luciferin very nearly completely precipitated (see p. 283).

The evidence from salting out experiments indicates that both luciferin and luciferase are proteins, the former on the border-line between proteoses and peptones, the latter a more complicated protein but not a globulin. Dubois[5] finds *Pholas* luciferin completely precipitated by saturation with $(NH_4)_2SO_4$ but not by $MgSO_4$ or NaCl.

Alcohol and Acetone.

If strong ethyl alcohol or acetone is added to a solution of crude luciferase, an abundant precipitate forms. This precipitate is found to contain the luciferase which is separated completely from solution by alcohol between 50 and 60 per cent, and by acetone between 70 and 80 per cent. We are dealing in both cases with a precipitation and not a coagulation of luciferase. The precipitates partially redissolve in water and if the solution is filtered luciferase is found in the filtrate. Indeed, the precipitates from addition of alcohol or acetone to crude luciferase may stand under alcohol (95 per cent) or acetone (90 per cent) respectively for 35 days without complete de-

struction of the luciferase, but there is always a diminution of activity which is no doubt eventually complete. The alcohol or acetone precipitate may be washed and dried and will give light whenever luciferin is added.

If strong ethyl alcohol or acetone is added to a solution of crude luciferin, a precipitate also forms, but it is not nearly so voluminous, since the heat-coagulable proteins are absent from the luciferin solution. With alcohol the precipitate is very fine; with acetone it is flocculent and clumps together readily. The precipitates washed once with 95 per cent alcohol and acetone respectively dissolve completely in water but these solutions give only a faint or no light if luciferase is added. On the other hand, the filtered alcohol and acetone solutions contain considerable amounts of luciferin. The absence of luciferin in the alcohol and acetone precipitates is only apparent. It is really partly precipitated by alcohol and acetone but is largely oxidized during the process of testing. Experiments indicate that oxyluciferin is present in the precipitates and can be reconverted into luciferin by appropriate methods. These experiments indicate that alcohol and acetone only partially precipitate luciferin, but completely precipitate luciferase. Boiling absolute alcohol will extract a considerable amount of luciferin and such a solution is quite stable even if exposed to air provided the alcohol is not allowed to evaporate. In alcohol-water and acetone-water mixtures the luciferin oxidizes readily and disappears in the course of a day or so.

Solubility in Organic Solvents.

The solubility of luciferin and luciferase in a number of pure organic solvents was tested by extracting the dried powdered *Cypridinæ*, filtering, and then testing the filtrate by the addition of luciferase and luciferin, respectively. Small pieces of the photogenic gland will sometimes pass through ordinary filter paper, but can always be recognized as isolated brightly luminous dots appearing when the solvent is tested for photogenic material. A heavy blotting paper holds back these fine particles and was used for filtering except in the case of very viscous solvents. The results are summarized in Table II.

It will be noticed that luciferase is not extracted by any of the solvents tried, whereas luciferin is soluble in a considerable number of

them. Methyl alcohol, ethyl alcohol, propyl alcohol, benzyl alcohol, ethyl acetate, glycerol, and glycol all dissolve a considerable amount of luciferin. Solvents non-miscible with water, such as benzyl alcohol or ethyl acetate, give up their content of luciferin to the water phase and it then luminesces if luciferase is present. In the case of the homologous series of aliphatic alcohols, the higher the alcohol in the series the less luciferin will it dissolve. The same is true for the homologous series of esters.

Luciferin is fairly stable in methyl alcohol, ethyl alcohol, propyl alcohol, benzyl alcohol, and glycerol if no water is present, but rather quickly disappears in acetone, glycol, and ethyl acetate, presumably because oxidation occurs more rapidly in the latter solvents. In the typical fat solvents as ether, chloroform, benzene, etc., the luciferin is insoluble. Luciferin is also soluble in glacial acetic acid but not in aniline.

There is nothing in the solubility relations of luciferase to indicate that it is not a protein. On the other hand, the solubilities of luciferin are certainly unusual for a natural protein. The best known class of proteins soluble in alcohol is the protamines of plants, but the protamines are insoluble in water and absolute alcohol. Zein, the protamine of corn, is soluble in 90 per cent ethyl alcohol, methyl and propyl alcohols, glycerol heated to 150°C., and glacial acetic acid.[6] Recently Osborne and Wakeman[7] have described a protein from milk having solubilities similar to those of gliadin, the protamine of wheat. Welker[8] has described a substance, obtained from Witte's peptone giving the biuret, Millon, and Hopkins-Cole tests, which is soluble in water and absolute alcohol but not in ether, and it is possible that others of the peptones are soluble in absolute alcohol. On the other hand, some proteins in the absence of salts form colloidal solutions in strong alcohol from which they may be precipitated by an appropriate salt.[9] As the absolute alcohol extract of *Cypridina* was made from dry material containing the salts of sea water, some salt was present, but there is always the possibility of sol formation.

[6] Osborne, T. B., *Ergebn. Physiol.*, 1910, x, 47.

[7] Osborne, T. B., and Wakeman, A. J., *J. Biol. Chem.*, 1918, xxxiii, 243.

[8] Welker, W. H., *Biochem. Bull.*, 1912–13, ii, 70.

[9] Private communication from T. B. Osborne.

If we extract dried *Cypridinæ*, which have previously been thoroughly extracted with benzene, with 800 cc. of boiling absolute alcohol for an hour, filter the alcohol extract through blotting paper and hardened filter paper, quickly evaporate the filtrate to dryness on the water bath, and dissolve the residue,in a small quantity of water saturated with CO_2, we obtain a yellow opalescent solution which gives a bright light with luciferase. This solution contains some protein or protein derivatives as it gives a very faint Millon reaction, a good positive ninhydrin test, reddish blue in color, but no biuret reaction. It precipitates with tannic and phosphotungstic acids but not with picric, acetic, trichloroacetic, or chromic acids or on saturation with $(NH_4)_2SO_4$. The phosphotungstic precipitate is not due to the presence of calcium. The extract gives a faint Molisch reaction for carbohydrates. As the evidence points to the presence of some protein material in the absolute alcohol extract of *Cypridinæ*, it is possible that this protein is luciferin. It should be emphasized, however, that the Millon reaction was very faint although the ninhydrin was quite marked.

Alkaloidal Reagents.

A solution of phosphotungstic acid added to crude luciferase solution gives a voluminous precipitate which, if washed quickly in running water on the filter and then suspended in water, does not completely dissolve but gives a good light if luciferin is added. It dissolves if a trace of dilute NH_4OH solution is added. The filtrate gives no light with excess luciferin, even though it contains some material which will precipitate on addition of more phosphotungstic acid.

Tannic acid added in slight excess to luciferase gives an abundant precipitate which contains luciferase and will give a good light with luciferin if suspended in water. It does not completely dissolve, even on addition of dilute NH_4OH solution. The filtrate gives a very faint light on addition of an excess of luciferin, even though an excess of tannic acid was used to precipitate and all precipitable material was thrown down.

Saturated aqueous picric acid added in equal volume to crude luciferase solution gives a not very abundant precipitate which if

filtered off, washed quickly with water, and suspended in water, does not completely dissolve. If luciferin is added the precipitate does dissolve and a good light appears. Dilute NH_4OH solution causes the precipitate to dissolve. The filtrate gives a very faint light when an· excess of luciferin is added, even though everything was precipitated that would precipitate with picric acid and an excess of the latter was present. The excess of picric acid was not sufficient to harm the luciferin used in testing.

Luciferase is, therefore, completely precipitated by phosphotungstic acid and almost completely precipitated by tannic and picric acids without harm.

Phosphotungstic acid added to a solution of crude luciferin to slight excess gives a bulky precipitate which can be filtered off and the filtrate is perfectly clear. It gives no further precipitate with phosphotungstic acid but contains a small amount of luciferin, as a faint light appears when luciferase in *excess* (since luciferase is also precipitated by the excess phosphotungstic acid present) is added. The precipitate washed on the filter with dilute phosphotungstic acid does not dissolve completely in water but partly dissolves forming an opalescent solution. It is brought into solution by adding crude luciferase or a trace of dilute NH_4OH solution. The precipitate contains luciferin and gives a brilliant light with luciferase. It is probably the slight alkalinity of the luciferase solution which causes resolution of the luciferin precipitate. The addition of a small amount of · HCl to the phosphotungstic-luciferin filtrate causes further precipitation, leaving a clear supernatant fluid containing no trace of luciferin.

Tannic acid likewise gives a voluminous precipitate with luciferin solutions and the filtrate is clear at first but may become cloudy in a short time, especially at the surface, unless considerable tannic acid is added. If an excess of tannic acid is present, as indicated by absence of further precipitate on adding more tannic acid, a small amount of luciferin is present in the filtrate but practically all is precipitated. The precipitate does not completely dissolve in water even if NH_4OH is added, but gives a bright light when luciferase is added. The addition of a small amount of acetic acid + NaCl or HCl does not cause further precipitation. Both the phosphotungstic

and tannic acid precipitates can be dried and will again give light with luciferase if redissolved.

TABLE III.

Properties of Photogenic Substances.

Property.		Luciferase.	Luciferin.
Alkaloidal reagents	Phosphotungstic acid...........	Completely pre-cipitated.	Very nearly com-pletely precipi-tated.
	" and acetic acids.		" " "
	" acid and HCl...		Completely precipi-tated.
	Tannic acid..................	Nearly completely precipitated.	Nearly completely precipitated.
	" and acetic acids.........		" " "
	" acid and HCl...........		" " "
	Picric "	Nearly completely precipitated.	Not precipitated.
	" and acetic acids.........		" "
	" acid and HCl...........		" "
	K₄Fe(CN)₆ and acetic acid......		" "
Heavy metal salts	Basic lead acetate...........	Completely pre-cipitated.	Not completely precipitated.
	Neutral lead acetate..........	Nearly completely precipitated.	" " "
	" " " and acetic acid......................		Not precipitated.
	Mercuric chloride.............	Not precipitated.	Not completely precipitated.
	" " and acetic acid......................		Almost completely precipitated.
	Uranyl nitrate and acetic acid..		Not completely precipitated.
Acids and alkalies	NaOH........................	Not precipitated.	Not precipitated.
	NH₄OH.......................	" "	" "
	Acetic acid....................	" "	" "
	H₂CO₃.......................	" "	" "
	Trichloroacetic acid.............	" "	" "

Picric acid added to luciferin solution gives no good precipitate but only an opalescence or turbidity. This is true even when picric acid

is present in considerable excess, as on addition of an equal volume of saturated aqueous picric acid. Further addition of a little dilute acetic acid or HCl does not cause precipitation. The solution filters turbid and luciferin may be demonstrated in the filtrate on adding luciferase. A yellow light due to the picric acid in solution appears. A little dilute NH₄OH clears the turbidity.

If we add acetic acid to luciferin solution until the precipitation (probably a precipitate of mucin and nucleoprotein) is complete and then dilute $K_4Fe(CN)_6$ solution, no further precipitate forms and luciferin is found abundantly in the filtrate which gives no further precipitate with $K_4Fe(CN)_6$ and acetic acid. These results are recorded in Table III.

Luciferin is, therefore, very nearly completely precipitated by phosphotungstic and tannic acids but not by picric acid. It is completely precipitated unharmed by phosphotungstic acid + HCl, but not by tannic acid + HCl or picric acid + HCl. It is not precipitated by potassium ferrocyanide and acetic acid. Dubois[10] finds *Pholas* luciferin to be completely precipitated by picric acid.

Heavy Metal Salts.

Some of the heavy metal salts (Pb acetate, $AgNO_3$, $CuSO_4$) precipitate in sea water. As both crude luciferin and luciferase solutions, although prepared with distilled water, contain small amounts of the salts of sea water, a slight precipitate would be formed upon addition of lead acetate. It is too small in amount to interfere with the action of lead acetate as a precipitant, but it must be borne in mind that some of the luciferin or luciferase might be adsorbed on any precipitate formed, as this is especially likely to occur with inorganic precipitates. Another difficulty encountered in precipitating protein solutions with heavy metal salts is that in excess of heavy metal salt some of the precipitate may redissolve. However, certain definite results were obtained by precipitating crude luciferin and luciferase with lead and mercury salts (the usual protein precipitants) and these are described below although their interpretation may be open to question.

[10] Dubois, *Ann. Soc. Linn. Lyons*, 1913, lx.

Neutral lead acetate solution was added to crude luciferase solution, drop by drop, until no further precipitate formed. The filtrate was clear and gave no further precipitate on adding more lead acetate. It gave a good light with excess of luciferin. The precipitate suspended in water does not dissolve, but upon addition of luciferin gives a bright light.

Basic lead acetate added drop by drop gives a bulky precipitate leaving a clear filtrate which gives no further precipitate on addition of more basic lead acetate. This filtrate gives no light with excess luciferin but the precipitate washed once with water on the filter gives a bright light.

Saturated $HgCl_2$ solution was added drop by drop to luciferase solution until no further precipitate occurs. The filtrate is perfectly clear and gives a bright light with luciferin although it gives no further precipitate with $HgCl_2$. The precipitate washed on the filter for 20 hours in running water is suspended in water. It does not dissolve but on addition of luciferin gives a fair light.

Luciferase therefore is completely precipitated by basic lead acetate, nearly completely precipitated by neutral lead acetate, but not precipitated by mercuric chloride.

If a saturated solution of $HgCl_2$ is added drop by drop to luciferin solution a precipitate forms. If this is filtered off, washed with dilute $HgCl_2$, and suspended in water, it does not completely dissolve but gives a good light if an excess of luciferase is added. The opalescent filtrate which gives no further precipitate with $HgCl_2$ gave a fair light on adding an excess of luciferase. Mercuric chloride and acetic acid almost completely precipitate luciferin from solution.

Neutral lead acetate solution added to luciferin gives a heavy precipitate. The solution filters cloudy at first even though no further precipitate forms on adding lead acetate, but soon filters clear. This clear filtrate containing some Pb gives a bright light on addition of excess of luciferase. The precipitate was washed several times on the filter and by decantation with dilute Pb acetate, and suspended in water. It does not completely dissolve but gives a good light with luciferase. Lead acetate and acetic acid give no good precipitate with crude luciferin.

Basic lead acetate gives a voluminous heavy precipitate with crude luciferin. After heating, the solution was filtered and the clear filtrate, which gave no further precipitate with basic lead acetate, gave a brilliant light with luciferase. The precipitate washed twice with water on the filter does not dissolve in water, but suspended in water gives a faint light on adding luciferase. Uranium nitrate + acetic acid does not completely precipitate luciferin from solution. These results are recorded in Table III.

Luciferin is therefore not completely precipitated from solution by mercuric chloride with or without acetic acid, neutral lead acetate or basic lead acetate, or uranium nitrate and acetic acid.

Acids and Alkalies.

As alkalies (KOH or NaOH in small, NH_4OH in greater concentration) precipitate the Mg of sea water, this salt should be removed from a crude solution of luciferin and luciferase before studying the precipitating effects of the substances. This can be done by the addition of a small amount of sodium pyrophosphate which forms Ca and Mg pyrophosphates insoluble in water. The precipitates are removed by filtration and the photogenic substances are found unharmed by the addition of pyrophosphate.

Neither luciferin nor luciferase is precipitated by addition of dilute NaOH or dilute NH_4OH to their crude solutions, first rendered free of Ca and Mg.

Dilute acetic acid added to luciferase solution gives a fine precipitate which is filtered off and washed with running water for 24 hours. It does not dissolve completely in water and gives only a faint light with luciferin. The precipitate is probably a mucin and carries down some adsorbed luciferase. The filtrate is perfectly clear, gives no further precipitate (sometimes a slight cloudiness on standing) with acetic acid, but a bright light with luciferin. The addition of slightly more acetic acid results in a clear filtrate giving no light with excess luciferin. The precipitate on the filter does give a faint light with excess luciferin but appears to be injured by the acid.

Saturation with CO_2 causes a precipitation in crude luciferase solution, but the filtrate gives a brilliant light with luciferin.

Dilute acetic acid added to concentrated luciferin gives a stringy precipitate. If filtered off, the filtrate is slightly opalescent but does not become more cloudy or precipitate if more acetic acid is added. It gives a bright light if luciferase is added to it. The precipitate does not completely dissolve in water, but if washed with water and suspended in water gives a fair light with luciferase. This precipitate is probably a mucin containing some adsorbed luciferin. In the luminous gland of *Cypridina* there is a material which stains as does mucin and it would be found in the hot water extract of the whole animal. The acetic acid precipitate does not form readily if the luciferin solution is hot when acetic acid is added. A little dilute NH₄OH will dissolve the precipitate and it can be reprecipitated by acetic acid. The filtrate from the acetic acid precipitation gives a voluminous precipitate with phosphotungstic acid which does not carry down all the luciferin unless a little HCl is also added, when luciferin is completely precipitated and may be demonstrated in the precipitate.

Dilute HCl alone gives a precipitate with crude luciferin solution but it almost completely dissolves in an excess of dilute HCl.

Saturation of a solution of crude luciferin in presence of some NaCl with carbon dioxide does not cause precipitation or an increase in turbidity.

Dilute trichloroacetic acid gives a stringy precipitate with crude luciferin solution similar to that with acetic acid, but abundant luciferin is found in the clear filtrate which gives no further precipitate with trichloroacetic acid. These results are recorded in Table III.

Hence neither luciferin nor luciferase is precipitated from crude solution by dilute NH₄OH or NaOH or by dilute acetic acid, and neither of them can belong to the group of histones (precipitated by dilute NH₄OH) or mucins, or nucleoproteins, or such phosphoproteins as caseinogen, which are precipitated by dilute acetic acid. A mucin or nucleoprotein is precipitated from both crude luciferase and luciferin solution by dilute acid and carries down some luciferase and some luciferin in the adsorbed state. It is easy to demonstrate that such an adsorption might occur. A solution of sodium caseinogenate mixed with either luciferin or luciferase and precipitated with acetic acid will carry down a considerable amount, but not all of the luciferin

or luciferase. Luciferin is not, but luciferase is injured by an excess of dilute acetic acid.

Dubois[5] reports that *Pholas* luciferin is not precipitated by carbonic acid in neutal solutions or by acetic acid except in presence of neutral salts and that it forms an insoluble alkali albuminate with NH_4OH. The latter is possibly a $Mg(OH)_2$ formed from the magnesium in the luciferin solution.

Adsorbents.

Proteins are usually separated from their solutions by one or another of the following methods: heat coagulation (in trace of acid); precipitation by alcohol or acetone (in large excess); precipitation by heavy metal salts (basic lead acetate, $HgCl_2$ and acid, uranium acetate and acid, etc.); alkaloidal reagents (phosphotungstic, tannic, picric acids, etc.); salting out (by $MgSO_4$ and acid, $(NH_4)_2SO_4$, etc.); digestion by proteolytic enzymes; adsorption (by chloroform, toluene, $Fe(OH)_3$, kaolin, and gum mastic).

We have already noted the behavior of luciferase and luciferin toward the first six methods. Both of these substances can also be separated from solution by adsorption on appropriate material; in fact, they are rather readily adsorbed especially by inorganic precipitates. Their complete adsorption is usually merely a matter of obtaining sufficient adsorbing surface area. For this reason comparative studies on adsorption of different materials are difficult to carry out because we cannot be sure of uniform surface area. However, it may be of interest to record a few of the experiments on adsorption.

A neutral dilute solution of luciferase was found to be completely adsorbed by bone-black, $Fe(OH)_3$, As_2S_3, infusorial earth (SiO_2), talc, and kaolin; nearly completely adsorbed by asbestos, pumice, $CaCO_3$, and $MgCO_3$; not nearly completely adsorbed by ground glass, sulfur powder, gelatin or agar-agar threads, heat-coagulated egg albumin, fresh precipitated caseinogen, cotton, or cornstarch.

A solution of luciferase shaken with five successive additions of fresh chloroform, until the chloroform is no longer emulsified but separates as readily as with water, is reduced considerably in luciferase content but the luciferase is not completely removed by the chloroform.

Neutral luciferin is completely adsorbed by bone-black, Fe(OH)$_3$, kaolin, talc, and CaCO$_3$, but not by many organic precipitates as caseinogen, cornstarch, or gelatin threads. There is the difficulty in studying adsorption of luciferin that oxidation may be accelerated by. the presence of finely divided material.

Luciferin can also be removed practically completely from solution by gum mastic according to the method of Michaelis and Rona[11] for removing proteins from blood serum.

CONCLUSIONS.

There seems to be very little doubt but that luciferase is a protein or so closely associated with proteins that their removal destroys its characteristic properties. The particular group of proteins to which it belongs may be arrived at by a process of exclusion, and only the group of albumins has properties which agree completely with those of luciferase.

Dubois believes *Pholas* luciferase to be an oxidizing enzyme similar to the oxydones of Battelli and Stern[12] because it is readily destroyed by fat solvents such as chloroform, strong alcohol, etc. He has detected iron in a luciferase solution which has dialyzed against running water for a long time, and believes it to be made up of protein in combination with iron and to act as an "oxyzymase ferrique."[5] *Cypridina* luciferase, on the other hand, is not *readily* destroyed by fat solvents. Toluene and chloroform are good preservatives, and I often make use of them for this purpose, keeping the luciferase solutions for many months. Professor A. H. Phillips of Princeton University has very kindly analyzed some whole dried *Cypridinæ* for me and finds iron, copper, and manganese but no zinc or vanadium to be present. Whether these metals are connected with the action of *Cypridina* luciferase is uncertain, but it is significant that all three of the metals thought to be concerned in organic oxidations are present.

Although a large amount of luciferin mixed with a small amount of

[11] Michaelis, L., and Rona, P., *Biochem. Z.*, 1907, ii, 219; Rona and Michaelis, *ibid.*, 1907, iii, 109; Michaelis and Rona, *ibid.*, 1907, iv, 11.

[12] Battelli, F., and Stern, L., *Biochem. Z.*, 1914, lxvii, 443.

luciferase will use up all the latter,[13] I agree with Dubois that luciferase has sufficient properties in common with the enzymes as a class to be considered an enzyme. The peroxidases are well known to be used up in the reactions they accelerate. All workers on enzymes agree that the more enzymes are purified the less active they become. The chemical procedures necessary to remove foreign material bring about irreversible changes in the enzyme itself, a characteristic also of many protein groups and of the colloidal state in general. This is true in the case of luciferase, for the crude luciferase solution is the most active preparation that can be obtained.

I believe that *Cypridina* luciferase should be placed in a class of oxidizing enzymes by itself—a group having the chemical reactions of an albumin, possibly in combination with some heavy metal, and which as far as we know, acts specifically on only one substance, *Cypridina* luciferin. It resembles the plant peroxidases in resisting the action of chloroform, toluene, etc., but will not oxidize any of the hydroxyphenol or aminophenol compounds[14] so readily oxidized by the

[13] If concentrated luciferin and weak luciferase are mixed, the light which appears will last a long time before going out. After the light disappears, if this mixture is diluted with water or more luciferin is added, no further luminescence occurs, but if more weak luciferase is added, light again is produced and lasts a considerable time. The fact that no more light appears on diluting the concentrated luciferin-weak luciferase mixture with water shows that the enzyme has not been inhibited by reaction products. If so, the dilution of these reaction products should allow the system to proceed to a new (false) equilibrium with production of light. Dubois (*Ann. Soc. Linn. Lyons*, 1917, lxiv, 105) has misunderstood my previous statement regarding this.

[14] Because of the ease with which many of these hydroxyphenyl compounds undergo autooxidation, one must always compare the color produced by luciferase solution with that produced in a control of boiled luciferase solution. I find that a concentrated luciferase solution well shaken with chloroform and filtered, which produced a brilliant light with luciferin, had no oxidative action on phenol, α-naphthol, *p*-phenylenediamine, ortol, orcinol, hydrochinon, resorcinol, pyramidon, phloroglucin, pyrocatechol, gallic acid, benzidine, pyrogallol, gum guaiac, amidol, tannin, or α-naphthylamine, either with or without H_2O_2. Dubois (*Ann. Soc. Linn. Lyons*, 1914, xli, 161) reports oxidation of pyrogallol, tannin, hydroquinone, guaiacol, Tromsdrof reagent, chlorohydrate of diaminophenol, phenylenediamine, and naphthol, naphthol B, and gum guaiac plus H_2O_2 by a solution of *Pholas* luciferase. These results are of little value, however, as there is no evidence that the oxidation is due to luciferase rather than the oxidizing enzymes which one finds in cell extracts of all animals whether luminous or non-luminous.

peroxidases, nor will the peroxidases oxidize luciferin with light pro duction. Dubois' researches show that *Pholas* luciferase differs in some properties from *Cypridina* luciferase, and my own work[15] indicates that firefly luciferase is more like that of *Pholas*. A comparative study of other species of luminous animals is needed in order to delimit more accurately the class of luciferases as a whole.

Luciferin presents many characteristics in common with the proteins, but two, which, to say the 'least, throw doubt on its protein nature: (1) its peculiar solubility (in alcohols, esters, and glacial acetic acid), (2) and its resistance to digestion by proteases, even by trypsin which has almost universal digestive action. These two peculiarities have been discussed above. We can only say that if a protein, luciferin must belong to a new group differing from *known natural* proteins in these respects. In general characteristics this new group would fall somewhere on the border-line between the proteoses and peptones. It would not be surprising to find in nature proteoses or peptones soluble in absolute alcohol. We know also that some NH-CO linkages of proteins are broken down 'with great difficulty by trypsin as it is difficult to obtain a tryptic digest of protein which does not give the biuret reaction, and the work of Fischer and Abderhalden has shown that certain artificial polypeptides are not digested by pure activated pancreatic juice. We have, then, three possibilities. Luciferin is (1) either a natural proteose not attacked by trypsin, or (2) if attacked by trypsin, its decomposition products (presumably amino-acids) still contain the group oxidizable with light production, or (3) it is not a protein at all. I believe that luciferin has too many protein characteristics to conform to the last possibility. I have been unable to oxidize with light production various mixtures of amino-acids (from beef and casein) by means of luciferase and consequently am led to believe that luciferin is a new natural proteose, soluble in absolute alcohol and not digested by trypsin.

Dubois believes *Pholas* luciferin to be a natural albumin with acid properties. *Cypridina* luciferin could not possibly be regarded as an albumin, but it is very likely that the luciferins of different species of luminous animals differ in certain characteristics. As in the case of

---

[15] Harvey, *Am. J. Physiol.*, 1917, xlii, 342.

he luciferases, we know that the luciferins are not identical sub-
stances, and only future work can determine in what particulars
hey differ.

A summary of the properties of *Cypridina* luciferin and *Cypridina*
uciferase will be found in the tables accompanying this paper.

THE TEMPERATURE COEFFICIENT OF PHOTOSYNTHESIS.

By W. J. V. OSTERHOUT and A. R. C. HAAS.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

The temperature coefficient of photosynthesis presents an interesting problem. On the one hand is the fact that light reactions have very low temperature coefficients; while on the other hand it appears that the temperature coefficients of photosynthesis reported by various investigators[1] are as high as those of ordinary reactions. It should be noted, however, that in a recent investigation van Amstel[2] found a temperature coefficient of 1.26, but expresses doubt whether this really represents the correct value for photosynthesis.

The difficulties of determining temperature coefficients are great in the case of land plants, for when leaves are exposed to sunlight their temperature rapidly rises and it may fluctuate as much as 10°C. in a half hour period. For this reason the writers have employed aquatic plants which form thin layers, the temperature of which can be sufficiently controlled.

In the present investigation the plants were placed in tubes filled with solution; these were immersed in a water bath the temperature of which was kept constant within 1°C., which sufficed for the purposes of the investigation. Thermometers placed in the tubes showed that the temperature did not rise more than 1°C. above that of the bath and that it did not fluctuate more than that of the bath itself. The plant employed in these investigations forms such thin layers

[1] Cf. Matthaei, G. L. C., *Phil. Tr. Roy. Soc.*, B, 1905, cxcvii, 47, and the interpretation by Kanitz, A., in Temperatur und Lebensvorgänge, 1915, p. 18. Also Blackman, F. F., and Matthaei, G. L. C., *ibid.*, 1905, lxxvi, 402. Blackman, F. F., and Smith, A. M., *ibid*. 1911, lxxxiii, 401.

The results of these observers have been interpreted by Brown and Heise as showing very low temperature coefficients (Brown, W. H., and Heise, G. W., *Philippine J. Sc.*, C., 1917, xii, 1).

[2] van Amstel, J. E., *Rec. trav. bot. néerlandais*, 1917, xiii, 1.

(0.078 mm. thick) that its temperature quickly reaches that of the surrounding liquid. This plant is the marine alga *Ulva rigida* (sea lettuce) which was chosen because of its rapid rate of photosynthesis, its hardiness under laboratory conditions, and its excellent mechanical properties.

The method[3] (which seems to be more accurate than those hitherto employed) consisted in placing the plant in sea water to which an indicator had been added, the whole being contained in a tube which was immersed to a depth of 2 or 3 inches in a water bath and exposed to direct sunlight.[4] Photosynthesis was allowed to proceed until a definite amount of CO_2 had been abstracted from the sea water, as shown by the color of the indicator. This was repeated as often as necessary. It has been shown in a previous article[3] that the rate of photosynthesis is not constant from the start, but steadily increases until it reaches a constant value. It was therefore necessary to continue the exposure until the constant value was reached. After determining the constant rate the temperature was changed and new determinations were made.

Since the amount of CO_2 abstracted by the plant (and consequently the amount of photosynthesis) was always the same, the rates at different temperatures are inversely proportional to the time required for the standard amount of photosynthesis.

Since the object of the investigation was to ascertain whether the temperature coefficients are those of light reactions or of ordinary chemical processes, it was not necessary to extend the experiments over a wide range of temperature. The temperatures chosen were 17° and 27°C., which are high enough to ensure rapid photosynthesis but which do not produce the slightest injury.

The results are shown in Table I. For convenience the rate of photosynthesis at 17°C. is taken as 100 and other rates are expressed as per cent of this. Thus the rate at 27°C. is found to be 169 ± 1.[5]

---

[3] The details of technique are fully explained in a previous article. See Osterhout, W. J. V., and Haas, A. R. C., *J. Gen. Physiol.*, 1918, i, 1.

[4] The tubes were inclined so as to receive the sunlight as nearly as possible at right angles.

[5] This figure represents the average of eight experiments. The results were first expressed as per cent and then averaged.

This is designated in the table as the apparent rate of photosynthesis since it is not corrected for respiration. The correction is made by adding the rate of respiration[6] to the apparent rate of photosynthesis. The sum gives the true rate of photosynthesis.[7] The true rate is seen to be 150 at 17°C. and 271 at 27°C., giving a temperature coefficient of 1.81.[8]

The question arises: How can the process have so high a temperature coefficient? The answer is not far to seek. The writers have suggested in a recent paper[9] that photosynthesis involves catenary reactions of the type $S \rightarrow M \rightarrow P$ in which S represents a substance which, under the influence of light, breaks down to form M;

TABLE I.

Temperature Coefficient of Photosynthesis and Respiration in Ulva.

	Temperature.		Temperature coefficient.
	17°C.	27°C.	
Apparent rate of photosynthesis...................	100	169±1.1	1.69
Rate of respiration.............................	50±1	102±1.5	2.04
True rate of photosynthesis......................	150	271	1.81

Each figure represents the average of eight experiments. The rate of photosynthesis at 17°C. (which is taken as 100) represents a change from pH 8.1 to pH 8.3 in 18.1 minutes.

this in turn forms P, the amount of which is proportional to the amount of photosynthesis. If the reaction $S \rightarrow M$ is more rapid than $M \rightarrow P$-

[6] This was ascertained by repeating the experiment with the same pieces of *Ulva* under precisely the same conditions except that light was excluded. It is expressed as per cent of the rate of photosynthesis at 17°C. (which is taken as 100).

[7] This involves the assumption that the rate of respiration is practically the same in light and in dark (as long as the temperature remains the same). There seem to be good grounds for this assumption.

[8] It is not likely that this result is affected by "limiting factors," since the light was practically full sunlight, and since the temperatures were near the optimum and the supply of CO_2 was sufficient, owing to the presence of carbonates and bicarbonates in the sea water. The effect of a limiting factor would be to make the coefficient appear less than it is in reality.

[9] Osterhout and Haas, *J. Gen. Physiol.*, 1918, i, 1.

(as is presumably the case), the speed of the process as a whole will depend chiefly on the speed of $M \rightarrow P$ (the velocity of the whole catenary process being regulated by the speed of the slowest member). It is therefore evident that when the temperature is raised its effect on the process as a whole will depend chiefly on its effect upon $M \rightarrow P$ rather than upon its effect upon $S \rightarrow M$. Hence if the light reaction $S \rightarrow M$ has a low temperature coefficient, while $M \rightarrow P$ (which is not a light reaction) has a high coefficient, the temperature coefficient of the process as a whole will be high. It is therefore not surprising to find that photosynthesis has a temperature coefficient of 1.81. Analogous cases exist in photochemistry.[10]

It is to be expected that similar relations will be found in heliotropic and heliotactic reactions where the stimulus is given by light and the growth or movement which follows is due to a slower process with a coefficient presumably higher than that of the light reaction.[11]

SUMMARY.

The temperature coefficient of photosynthesis in *Ulva* (between 17° and 27°C.) is 1.81. This may be explained by assuming that the process involves a light reaction with a low coefficient followed by an ordinary reaction with a high coefficient.

[10] Cf. Bovie, W. T., *Science*, 1913, xxxvii, 373.

[11] According to T. Nybergh (*Ber. bot. Ges.*, 1912, xxx, 542) this is the case for the oat seedling, but this is disputed by Marie de Vries (*ibid.*, 1913, xxxi, 233; *Versl. wis. en natuurk. Akad. Wetensch. te Amsterdam*, 1913, xxi, 1056.) In certain cases the whole process may depend on the diffusion of a substance (produced in the light) from the point of origin to another region. The coefficient may then be that of diffusion (usually not over 1.28).

A COMPARATIVE STUDY OF PERMEABILITY IN PLANTS.

By W. J. V. OSTERHOUT.

(*From the Laboratory of Plant Physiology, Harvard University, Cambridge.*)

The writer has arrived at certain general conclusions concerning the behavior of protoplasm, which are based largely on the study of a single plant. To ascertain whether they have general validity it is necessary to study other plants as well as animals. The following paper gives a brief résumé of results obtained with a red alga (*Rhodymenia palmata*), a green alga (*Ulva rigida*), and a flowering plant (*Zostera marina*). These forms were chosen because they differ greatly from the brown alga *Laminaria Agardhii* (which furnished the material for the experiments hitherto described), not only in the chemical composition of the cell wall[1] but also in the structure of their tissues.

The experiments on *Laminaria* were made by cutting disks from the fronds and packing them in a cylinder, the electrical resistance of which was determined as previously described.[2]

The experiments with *Rhodymenia palmata* (the common dulse of the Atlantic coast) were carried out in precisely the same manner as those on *Laminaria*. This was possible because *Rhodymenia* forms thin sheets of tissue, large enough to be cut by a cork borer into disks 13 mm. in diameter. The disks were packed together, like a roll of coins, to form a cylinder, the electrical conductivity of which was measured as previously described.[2] *Rhodymenia* is much more difficult to keep in good condition than *Laminaria*, and on this account is not so well suited to investigations of this kind. It demands a low temperature and thorough aeration, and even with these does not live so long as *Laminaria*.

---

[1] Regarding the composition of the cell wall in algæ see Czapek, F., Biochemie der Pflanzen, Jena, 2nd edition, 1913, i, 640 ff.

[2] Osterhout, W. J. V., *J. Biol. Chem.*, 1918, xxxvi, 557.

In *Ulva* the structure of the frond is much simpler than in the other plants studied. It consists of two layers of similar cells, forming a membrane about 0.078 mm. in thickness. The cell walls are thin and consist of cellulose.[3] As it is not sufficiently stiff to be treated like *Laminaria*, pieces were cut out and supported between disks of hard rubber and celluloid as described in a previous paper.[4]

The leaves of *Zostera* have a very simple structure. The fibro-vascular bundles are poorly developed. There are intercellular spaces, but it is possible to find leaves in which there is very little gas in these spaces. In such material the amount and position of the gas seems to remain unchanged during the experiment. The cell walls are of cellulose.

As the leaves are not wide enough to furnish disks such as were used in other experiments, a different procedure was adopted. A number of pieces were held in a horizontal position (parallel and a little separated) by means of two parallel celluloid combs placed at right angles to the length of the leaves. The electrode carriers moved between the combs, forcing the leaves together when measurements were made. The electrode carriers were held in frames as in the case of *Laminaria*, but the two upper glass rods were removed and a special form of hard rubber plate was placed at each end.

In some of the experiments pieces of the leaf were placed between disks of hard rubber and celluloid, just as in the case of *Ulva*.

In all cases the resistance was first determined in sea water, which was then replaced by another solution of the same conductivity. All readings were made at the same temperature or, if necessary, corrected to the proper temperature.

Experiments on *Laminaria* have shown that it is possible to follow the progress of death in the same manner as the progress of chemical reactions *in vitro*. For the most part the process follows more or less closely a monomolecular course. For *Laminaria* placed in NaCl 0.52 M, the velocity constant of the reaction at 15°C. was found (as the average of eight experiments) to be 0.00723. For *Rhodymenia* the same number of experiments (at the same temperature) gave $K = 0.0009$. Hence the rate appears to be about eight times as great

[3] The wall contains a methyl pentosan yielding rhamnose. Cf. Czapek,[1] 641.
[4] Osterhout, *J. Biol. Chem.*, 1918, xxxvi, 563, fig. 3.

in the case of *Laminaria*. In *Ulva* the rate is slower than in *Rhodymenia*, but *Zostera* is in this respect intermediate between *Laminaria* and *Rhodymenia*.

Up to a certain point the death process in *Laminaria* is reversible. Thus tissue remaining in NaCl 0.52 M until it has lost 10 per cent of its resistance will recover if replaced in sea water. This is also true of *Ulva*, *Rhodymenia*, and *Zostera*.

According to their effect upon *Laminaria* substances may be divided into two classes: (1) those which (like NaCl) cause only a fall in resistance, and (2). those which (like CaCl₂) cause a rise of resistance followed by a fall. There are great differences among these substances in respect to the effects which they produce, but as far as the experiments have gone these differences are similar in all the plants studied. Thus it is found that the amount of rise in resistance and its duration are much less in the case of Mg than of Ca and this applies to all the plants mentioned.

Rhodymenia agrees with *Laminaria* in showing a rise in resistance[5] in CaCl₂, BaCl₂, SrCl₂, MnCl₂, NiCl₂, and a greater rise in alum, Ce(NO₃)₃, and La(NO₃)₃.

Ether produced a rise in *Laminaria* and *Ulva* (*Zostera* was not studied). In *Rhodymenia* ether (2.5, 3, 5, and 5.5 per cent by volume), chloroform (0.02, 0.03, and 0.05 per cent by volume), and alcohol (1, 3.5, 7, 8 per cent by volume) added to the sea water produced little or no rise. This is not surprising in view of the fact that these substances always produce less rise in *Laminaria* than does Ca and that even Ca produces very little rise in *Rhodymenia* (Fig. 1). In respect to recovery from the injury caused by these substances *Rhodymenia* agrees with *Laminaria* in that recovery is practically complete in alcohol (if the fall in resistance has not gone too far), but is almost entirely absent in ether and chloroform.[6]

In general it was found that substances of one class antagonize those of the other, not only in the experiments on *Laminaria*,[7] but also in the cases of the other plants mentioned. As was to be expected, the most favorable proportions were not always the same for

[5] Osterhout, *Bot. Gaz.*, 1915, lix, 317, 464.

[6] Osterhout, *Bot. Gaz.*, 1916, lxi, 148.

[7] Osterhout, *Science*, 1915, xli, 255.

the different plants. Thus it was found that in the case of *Rhodymenia* it required more Ca to antagonize Na than it did in the case of *Laminaria*. It was also observed that in the case of *Rhodymenia* (Fig. 2) the antagonism was not so great as in *Laminaria* and this appears to be correlated with the fact that less decrease of permeability is produced by Ca in *Rhodymenia* (Fig. 1). · In other words, the effect of such a substance as Ca upon permeability not only indicates
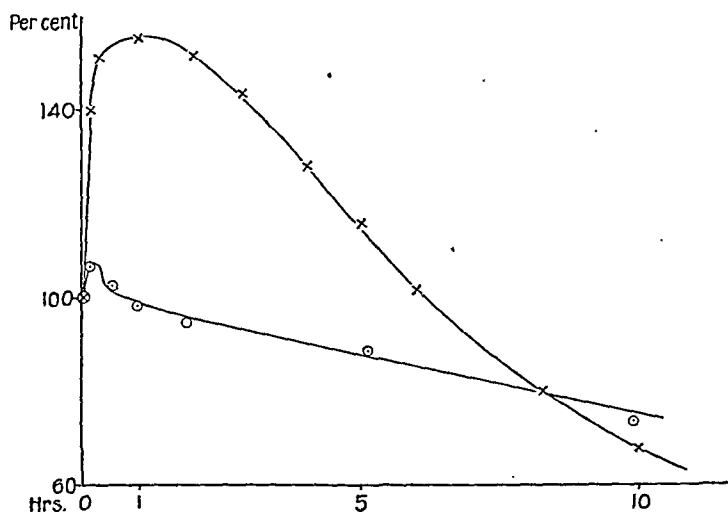

FIG. 1. Curves showing the effect of CaCl$_2$ 0.278 M on the electrical resistance of *Laminaria* (upper curve) and of *Rhodymenia* (lower curve). The ordinates denote net electrical resistance expressed as per cent of the normal resistance in sea water (which is taken as 100 per cent). Temperature 17° ± 2°C. Average of six experiments. Probable error less than 3 per cent of the mean.

what substances it will antagonize, but also, to some degree at least, the amount of antagonism.

It may be added that *Rhodymenia* affords an interesting confirmation of the value of the electrical method in measuring antagonism since the plants begin to change color soon after injury occurs. It was found that the relative rates of death as indicated by color changes in NaCl, CaCl$_2$, and the various mixtures corresponded with the results obtained by determining conductivity.

One of the most interesting experiments on *Laminaria* consists in placing it first in NaCl (causing a fall in resistance) and then in CaCl₂, causing a rise. In this way rapid changes of permeability may be produced and the alternation may be continued for a considerable
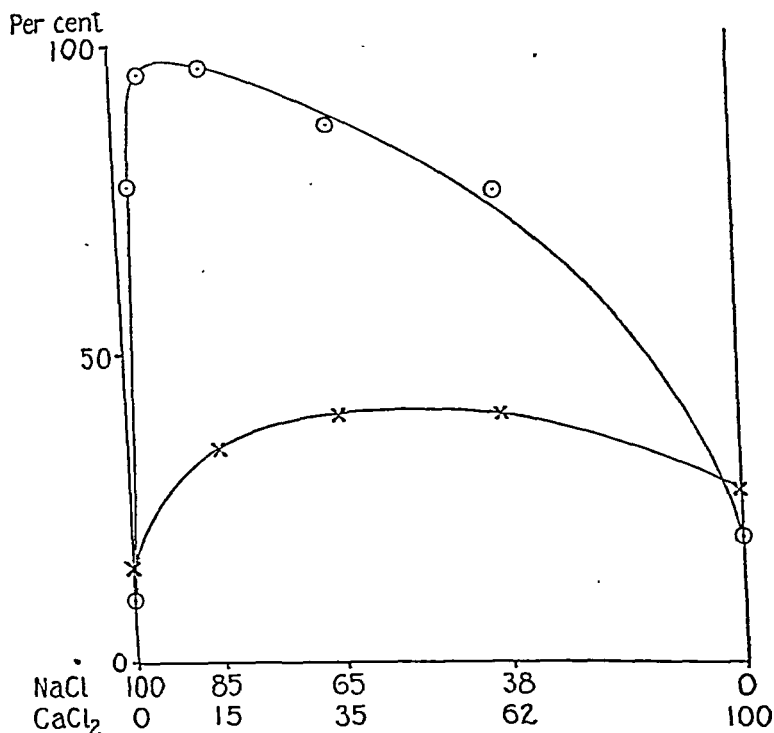

FIG. 2. Curves showing antagonism (after an exposure of 24 hours) between NaCl 0.52 M and CaCl₂ 0.278 M in *Laminaria* (upper curve) and *Rhodymenia* (lower curve). The ordinates denote net electrical resistance expressed as per cent of the normal resistance in sea water (which is taken as 100 per cent). The abscissæ denote molecular proportions of the solutions (all the solutions having the conductivity of sea water). Thus NaCl 85, CaCl₂ 15 signifies a mixture of 75 cc. NaCl 0.52 M + 25 cc. CaCl₂ 0.278 M in which the molecular proportions of Na to Ca are as 85 to 15. Temperature 17.5° ± 5°C. During the 24 hours the resistance of *Laminaria* in sea water remained practically unaltered while that of *Rhodymenia* fell to 84.5 per cent. Average of six experiments. Probable error less than 5.2 per cent of the mean.

period without any bad after effects.[8]   This is also the case with the other plants studied.

It appears that in all the plants investigated there is a striking agreement in essentials, though there is considerable diversity in details.   It is therefore evident that the conclusions drawn from the study of *Laminaria* are of general validity for all the plants investigated.   It is hoped that similar studies on animals may be presented in the near future.

SUMMARY.

Quantitative studies on *Laminaria* (a brown alga), *Ulva* (a green alga), *Rhodymenia* (a red alga), and *Zostera* (a flowering plant) show that the behavior of these plants, in respect to changes in permeability, is essentially alike in all cases.

[8] Osterhout, *Bot. Gaz.*, 1915, lix, 242.

NATURE OF THE RETARDING INFLUENCE OF THE THYMUS UPON AMPHIBIAN METAMORPHOSIS.

By EDUARD UHLENHUTH.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

There is a general agreement concerning the influence of the thyroid pon the metamorphosis of anuran amphibian larvæ. All authors rho reported experiments on feeding thyroid to tadpoles, regardless f the locality in which they were carried on and on whatever species f anura they were performed, invariably found that thyroid feeding .lways resulted in a precocious metamorphosis. The experiments on .hymus feeding, however, are characterized by just the opposite eature, the results being very inconstant. Thymus feeding some-:imes resulted in retardation or entire prevention of metamorphosis; ;ometimes it had no effect as compared with the controls. Such different action not only was obtained in the experiments of different authors, performed on different species and at different places (Gudernatsch obtaining positive, Swingle negative results), but even when the same investigator experimented on the same species different individuals were affected differently by the thymus diet (Romeis). Nevertheless, it is certain that in some cases thymus feeding actually prevented metamorphosis and resulted in giant larvæ, while no such effect was obtained in the controls on the normal diet.

It seems necessary therefore to explain why thymus feeding sometimes does and sometimes does not prevent metamorphosis.

The writer performed a large number of experiments on the larvæ of *Ambystoma maculatum,*[1] *Ambystoma opacum*, and *Ambystoma tigrinum*, and it was found that while the thyroid contains a specific substance enforcing metamorphosis, the preventive effect of thymus feeding is due to the absence from the thymus of a substance necessary for the formation or excretion by the thyroid of the substance causing metamorphosis.

[1] For nomenclature of salamanders see Stejneger, L., and Barbour, T., A check list of North American amphibians and reptiles, Cambridge, 1917.

Inhibitory Effect of the Thymus.

Some experiments may be reported briefly showing that thymus feeding sometimes actually prevents metamorphosis, though in the majority of the larvæ it has no such effect.

In a set of larvæ of *Ambystoma maculatum*, kept at approximately 15°C., fed on tubifex and earthworms, and consisting of about 100 specimens, most of the larvæ, which had hatched in the beginning of May, 1916, metamorphosed during the months of August and September. On October 21, eight of these were still larvæ. These were picked out to form a series of normal controls (c. W. 1916). In a set of eighteen larvæ of the same species and of the same age and kept under the same conditions as the above series, but fed on thymus soon after hatching, nine larvæ had metamorphosed up to October 21. The remaining nine were picked out to form a thymus-fed series (c. T. 1916). The worm-fed larvæ of Series c. W. 1916 metamorphosed at an age of 28 weeks and 6 days on an average; the first larva to metamorphose was 25 weeks and 4 days old at the time; the last one 33 weeks and 4 days. Among four larvæ of the thymus-fed Series c. T. 1916, the first animal was 27 weeks old when it metamorphosed, the last one 55 weeks and 2 days. Of these four larvæ the average age at the time of metamorphosis was 35 weeks and 2 days. The other five larvæ of the thymus-fed series died before metamorphosis; three at the age of 31 weeks and 2 days, one at the age of 40 weeks and 3 days, and one at the age of 62 weeks and 3 days. Thus one animal of the thymus-fed series remained larval for about 1 year and 3 months; at this time it did not show any signs of metamorphosis and it seems possible that it would have remained permanently in a larval state. The writer so far has never observed in his normal controls individuals which remained larval for so long a time. Thus there can be no doubt that in this thymus-fed series metamorphosis was retarded as compared with the controls and in one case probably was even prevented.

In a series of eight larvæ of *Ambystoma opacum*, which were kept at about 25°C. and fed moderately on earthworms, the average time of metamorphosis was 26 weeks; in a series of eight larvæ of the same species of the same age and from eggs of the same female as the

larvæ of the foregoing series, the larvæ were fed on thymus and with the largest possible amount of this diet; otherwise they were kept like the worm-fed controls. The average time of metamorphosis in this series was 17 weeks and 5 days; *i.e.*, less than in the worm-fed series. In this number, however, two larvæ are not included, both of which died before metamorphosis; one of them reached the age of 31 weeks and 2 days, the other 34 weeks and 1 day without having metamorphosed. Thus in this series again a considerable delay (if not an inhibition) of metamorphosis was produced by the thymus diet in two animals.

A third species, *Ambystoma tigrinum*, was experimented on. This species, as mentioned in a recent publication[2] shows the least effects when fed on thymus. Among six animals kept at approximately 25°C. and fed on thymus, the larvæ metamorphosed simultaneously with the controls. Among six other animals kept at approximately 15°C. and fed on thymus, only five larvæ metamorphosed simultaneously with the controls (between 22 and 26 weeks after hatching), while one individual though now over 74 weeks old is still in larval condition. Thus also in this species thymus feeding apparently resulted in a considerable retardation of metamorphosis though only in one individual.

From these experiments on urodelan larvæ it is again evident that the effect of the thymus is extremely variable. But it is also a fact that in some of the larvæ metamorphosis was retarded by the thymus diet and in two probably completely prevented. Further experiments, therefore, were carried out to determine this point.

Inhibitive Effect of Thymus upon Amphibian Metamorphosis Is a Deficiency Phenomenon.

It is clear that the considerable variability of the action of the thymus cannot be explained on the assumption that the inhibitive effect of that gland is due to the presence of a specific inhibiting substance in the thymus. But it can be explained if this effect is due to the absence from certain parts of the thymus of a substance necessary to produce metamorphosis and which is contained, in minute quan-

[2] Uhlenhuth, E., *J. Gen. Physiol.*, 1918. i. 23.

tities, in other parts of the thymus, in normal food, and perhaps in the water of certain localities where unsuccessful experiments on thymus feeding have been carried out.

If the preventive influence, which the thymus exhibits in some of the larvæ, is due to the presence of some specific metamorphosis-inhibiting substance, metamorphosis evidently should be prevented by the thymus even if normal food is added to the thymus diet. This is the case, for instance, with the metamorphosis-producing substance of the thyroid gland. Lenhart[3] has shown that if a certain amount of the active substance of the thyroid, able to produce accelerated differentiation and not large enough to result in death from emaciation before differentiation can take place, is introduced into the organism, differentiation will be accelerated and at a definite rate, whether the tadpoles are fed only on thyroid or whether some other food (liver) is added to the glandular diet. In fact, it seems, from all experiments so far performed with thyroid, that it is of no importance what food the larvæ receive; the addition of only a minute quantity of thyroid substance causes metamorphosis at an accelerated rate. We found the same to be true for the larvæ of salamanders. Young (5 weeks old) larvæ of *Ambystoma opacum* which were fed on earthworms, were placed in a 0.02 per cent solution of iodothyrin; in spite of the earthworm diet and of the small quantity of thyroid substance used (Bayer's iodothyrin), all larvæ metamorphosed from 8 to 9 days after the commencement of the thyroid treatment, while the controls needed 7 to 8 weeks more to metamorphose. The thymus itself contains a specific substance which is highly toxic and produces tetany in the larvæ of *Ambystoma maculatum* and *opacum* as described in a former article.[2] The action of this substance as regards its constancy is quite similar to the action of the metamorphosis-producing substance of the thyroid and very unlike the metamorphosis-inhibiting action of the thymus. No matter what food is added to the thymus diet larvæ fed on thymus always had tetany. And like the thyroid substance, the tetany toxin of the thymus is also characterized by the constancy of its action; it produces tetany in each individual.

[3] Lenhart, C. H., *J. Exp. Med.*, 1915, xxii, 739.

It is quite different with the prevention of metamorphosis by the thymus. If normal food is added to the thymus diet, metamorphosis will take place in each individual at the same time as in the controls. This is shown in the following experiments on larvæ of *Ambystoma maculatum* of the same age, from eggs of the same female, and all kept at approximately 25°C. One series of 30 larvæ was fed only on worms, one series of 15 larvæ only on thymus, and one series on thymus and worms alternately. Fig. 1 shows the result. The larvæ
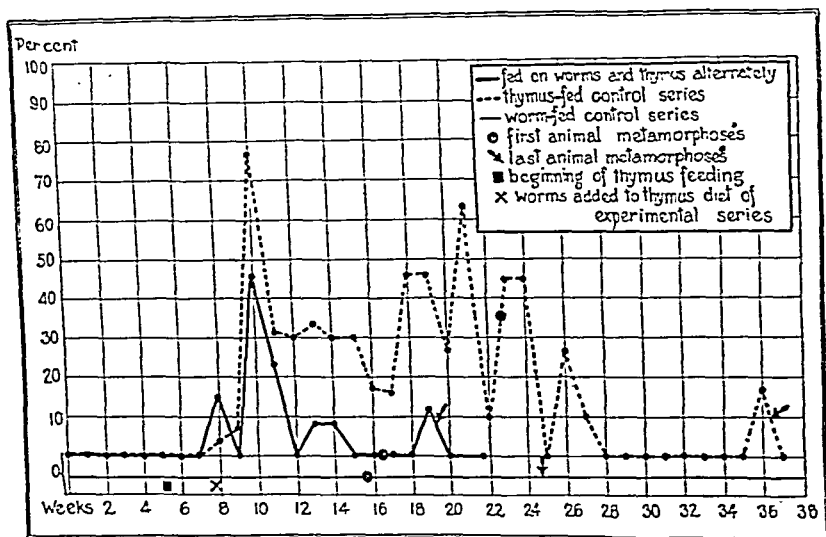

FIG. 1. Effect of worm diet when added to thymus diet upon metamorphosis and tetany in *Ambystoma maculatum* larvæ. Curves indicate percentage of tetanic animals.

of the mixed food series metamorphosed simultaneously with those of the control series, which were fed on worms only; while in the series fed on thymus, metamorphosis started when all animals of the series fed on worms and on worms and thymus alternately had already metamorphosed, and the last larval animal died about 12 weeks later without having metamorphosed at all. In other words, in the series fed on mixed diet the food added to the thymus diet contained the substance which is necessary for metamorphosis and which is lacking in the thymus; and thus this substance was introduced into

the larval organism in such a large amount that metamorphosis occurred in this series simultaneously with the controls. The ability of the normal food to counteract the inhibitive effect of the thymus upon metamorphosis was the more conspicuous as the same food was quite ineffective in preventing the tetanic convulsions produced by the tetany toxin present in the thymus; this may be seen from the curves which indicate the percentage of tetanic animals found at the time of observation among the thymus-fed and mixed food series.[4]

That the action of the thymus is merely due to the absence of the substance necessary to produce metamorphosis, is also demonstrated in experiments in which thymus-fed larvæ are placed in a solution of thyroid substance. If the thymus contained a specific metamorphosis-preventing substance, one would expect an antagonistic neutralization of the thyroid substance by the thymus substance. But instead the thyroid substance even when present in minute quantities induces prompt metamorphosis in the thymus-fed animals. For the sake of illustration one experiment may be reported here. A set of six thymus-fed larvæ of *Ambystoma opacum* was fed on thymus. At an age of 6 weeks the larvæ were placed in a 0.02 per cent solution of iodothyrin which after about 20 hours was replaced by a 0.006 per cent solution. 8 days after the beginning of the thyroid treatment all the larvæ were metamorphosed although fed on thymus, while the controls not treated with iodothyrin needed from 6 to 7 weeks more to metamorphose. In this series again we observe that the effects of the thymus, which actually are due to the presence of a special[5] substance in the thymus, are not stopped by the thyroid treatment. The larvæ exhibited severe tetanic convulsions caused by the tetany toxin of the thymus; these convulsions occurred in spite of the thyroid treatment with undiminished strength.

[4] For detailed discussion see Uhlenhuth, *J. Gen. Physiol.*, 1918, i, 33.

[5] The term specific in connection with the active principle of the thyroid gland has been avoided here, for it does not seem to be proved definitely that the effects exerted by the iodothyrin cannot be brought about by any other substance or any other factor. Since the term specific refers not only to the origin of the inner secretory substances but also to their effects, it is misleading in connection with the thyroid substance.

DISCUSSION.

From the above experiments it is evident that an exclusive thymus diet sometimes can retard or even prevent metamorphosis. But while the ability of the thyroid to enforce metamorphosis is due to the presence in the thyroid of a special substance, the inhibitory action of the thymus is due to the absence of a substance without which metamorphosis is impossible.

In order to appreciate this fact fully we must remember the experiments performed by Allen[6] and Hoskins,[7] which have demonstrated that tadpoles whose thyroid glands have been extirpated are unable to metamorphose. This means that under normal conditions at the time of metamorphosis the thyroid begins to excrete the metamorphosis-producing substance which under experimental conditions is introduced into the organism by feeding thyroid to the larvæ or keeping them in a solution of thyroid substance. Since under normal conditions no thyroid is fed to the animals and since it is the thyroid of the animal itself which excretes the substance in question, we must assume that the normal food of the larvæ contains a substance which is necessary to develop the thyroid of the larva to a state in which it can excrete the metamorphosis-producing substance. Whether or not the substance necessary to develop the thyroid and furnished in normal conditions by the normal food of the larvæ is identical with the metamorphosis-producing substance excreted later on by the thyroid, cannot be decided at present; but in this respect the attempts made by Allen[6] to enforce metamorphosis of thyroidless larvæ by feeding them on thyroid are important. If it is possible to enforce metamorphosis in thyroidless larvæ by feeding them on thyroid, but impossible by feeding them normal food, the substances contained in the normal food are able to develop the thyroid to the excreting stage, but they are unable to evoke metamorphosis in the absence of the thyroid. and, therefore. they are not identical with the thyroid substance. The results so far obtained by Allen point in the latter direction. It is this substance, necessary to develop the secretory stage in the thyroid. which is missing in the thymus.

[6] Allen, B. M., *Science*, 1916, xliv, 755; *J. Exp. Zool.*, 1917–18. xxiv, 499.

[7] Hoskins, E. R., and Hoskins, M. M., *Proc. Soc. Exp. Biol. and Med.*, 1917–18, xv, 102.

The question arises now in which way do some of the thymus-fed larvæ procure enough of the substance required for the development of the thyroid, while other thymus-fed larvæ are unable to obtain enough of it though all of these larvæ apparently are fed on an equally exclusive thymus diet? Although at present an exact statement on this matter is impossible we must seek its explanation in the fact that evidently the amount required is so small that it was difficult in our experiments so far performed to control the sources which sometimes supplied this substance against our will. As to the actual source of the substance it is possible that the connective tissue constituting the septa between the lobules of the thymus may contain some of it; in fact, this is probable since the septa are in no way a specific tissue like the rest of the thymus. The possibility of some of the larvæ having obtained, by chance, more of the septa than others must be admitted. In this way the great variability of the results of the same author could be explained. There is also a possibility that the water may contain some of that substance; in favor of this would be the fact that some authors, like Swingle,[8] did not obtain any retarding effects at all in their thymus-fed series. Of course, the difference in the reaction of different individuals of the same series of one experimenter can be explained less readily on that possibility.

Our experiments suggest the possible character of the influence of environmental factors on metamorphosis. The problem of amphibian metamorphosis, as well as the problem of internal secretion, assumes a new shape in the light of that fact. On the one hand, it has become clear from the experiments of Allen and his followers that metamorphosis is directly dependent on the action of a certain inner secretory gland of the amphibian larva; on the other hand, it is evident that the development of the secretory stage of that inner secretory gland depends ultimately on certain purely environmental, non-glandular factors. And it is now time that we should recall such attempts as those made by Duméril[9] and von Chauvin[10] to enforce or prevent metamorphosis by purely external non-glandular

[8] Swingle, W. W., *J. Exp. Zool.*, 1917–18, xxiv, 521.

[9] Duméril, A., *Ann. sc. nat., Zool.*, 1867, vii, 229.

[10] von Chauvin, M., *Z. wiss. Zool.*, 1885, xli, 365.

factors; these attempts in the case of von Chauvin doubtless were successful, though the actual relation between the factors employed and the result obtained cannot well be understood at present.

SUMMARY.

1. Though thymus-fed salamander larvæ often metamorphose normally, thymus feeding sometimes retards and in rare cases inhibits metamorphosis completely.

2. The addition of normal food to a thymus diet abolishes the inhibitory effect of the thymus.

3. Addition of a small quantity of iodothyrin leads rapidly to precocious metamorphosis of thymus-fed larvæ.

4. The inhibitory effect of the thymus is not due to a specific inhibiting substance in the thymus, but to the absence from the thymus of a substance required to develop the thyroid to the secretory state.

PARATHYROIDS AND CALCIUM METABOLISM.

By EDUARD UHLENHUTH.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

MacCallum and Voegtlin,[1] as well as other authors, have found that in tetany resulting from the extirpation of the parathyroids the Ca content of the blood and the organs (brain) is greatly reduced and that the introduction into the organism of Ca salts, subcutaneously, intravenously, or *per os*, suppresses the tetanic convulsions of the animals operated on. These findings have been confirmed recently by Howland and Marriott[2] in tetany of children. Spontaneous tetany in human beings has apparently the same cause as parathyreoprival tetany, both being due to the non-functioning of the parathyroids. Furthermore, it is known[1] that after parathyroidectomy, tetanic convulsions may be suppressed by bleeding the animals and substituting the amount of blood drawn by an equal amount of salt solution. From the latter fact MacCallum and Voegtlin conclude that in the absence of the parathyroids some toxic substance accumulates in the blood, which normally is antagonized by the parathyroids. They assume further that the toxicity of this substance is due to its ability to combine, in some unknown way, with calcium which it extracts from the organs, causing its excretion and thereby diminishing the Ca content of the blood and organs. MacCallum thinks that the muscular convulsions in tetany are the result of the diminution of the Ca concentration, the function of the parathyroids being to regulate the Ca concentration by antagonizing the toxic substance and thus preventing it from extracting the Ca salts from the body.

As regards the existence of a toxic substance involved in the causation of tetany, the writer has shown that such a substance actually

---

[1] MacCallum, W. G., and Voegtlin, C., *J. Exp. Med.*, 1909, xi, 118.

[2] Howland, J., and Marriott, W. McK., *Bull. Johns Hopkins Hosp.*, 1918, xxix, 235.

exists and is contained in the thymus gland.[3]   In the present article certain experiments will be reported demonstrating that calcium is able to suppress the tetanic convulsions, at least to some extent; the writer, however, was unable to convince himself that this effect upon tetany is characteristic for the calcium and furthermore the experiments in question, though they do not exclude a possible relation between the toxic substance and the calcium, prove conclusively that, as far as the animals used in these experiments are concerned, the tetany toxin, even in the presence of the calcium and in the absence of convulsions, brings about severe lesions of the muscular system resulting probably from lesions of the central nervous system caused by the tetany toxin and not prevented by the calcium.

<center>EXPERIMENTAL.</center>

In order to test the action of Ca lactate upon tetanic animals, a number of larvæ of the salamander *Ambystoma opacum* were fed on thymus and kept at the same time in a solution of Ca lactate in ordinary tap water; another set of larvæ of the same age and from the same female were kept in a solution of Mg lactate of the same concentration as the Ca lactate solution.   A series of larvæ from a different female, fed on thymus, but kept in ordinary tap water, served as controls; since differences between larvæ from different females as regards the severity of tetany when fed on thymus are so small as to be negligible, the error introduced by comparing larvæ of different females is very small.   For each of the three thymus series one series was kept as control, in which all conditions were the same as in the thymus-fed series, except that small pieces of earthworms instead of thymus served as food.   None of the worm-fed control series developed tetany.

I.   Thymus-Fed, Untreated Controls (Fig. 1, Curve I).—Six larvae of *Ambystoma opacum* were fed on thymus exclusively.   As usual the tetanic attacks began after the larvæ had reached a certain developmental stage[3] and soon reached a maximum.   Each single individual came down with tetanic convulsions.   When metamorphosis was

[3] Uhlenhuth, E., *J. Gen. Physiol.*, 1918, i, 23, 33.

approached, tetanic convulsions ceased, and after metamorphosis all larvæ were free from it.

Besides the tetanic convulsions the other symptoms of tetany were also present in all animals; the legs and feet became permanently twisted and contracted and the entire body assumed the shape char-

FIG. 1. Tetanic convulsions in a thymus-fed series of *A. opacum* (Curve I); in a thymus-fed series, kept in Ca lactate (Curve II); and in a thymus-fed series, kept in Mg lactate (Curve III). The ordinates indicate the percentage of animals showing tetanic convulsions at the time indicated on the abscissæ. Convulsions of the entire body and convulsions of the posterior portion of the body alone are considered.

acteristic of tetanic animals. The limbs and most of the muscles necessary for the movements of the animals became permanently paralyzed.

II. Thymus-Fed Series, Kept in Ca Lactate (Fig. 1, Curve II).— Seven larvæ of *Ambystoma opacum* were used, one of which died soon after the experiment had been started. Several days after the thy-

mus feeding had been started, one of the larvæ had a tetanic attack. 8 days after the beginning of thymus feeding the larvæ were transferred to a 1/1250 M solution of Ca lactate in tap water; this suppressed tetanic convulsions up to the 7th week, while in the untreated controls tetany had started at the end of the 5th week. Though the concentration of the solution was lowered to only 1/2500 M Ca lactate, the tetany curve did not rise as it did in the controls, but remained low until at the end of the 10th week. The concentration of the solution was increased to 1/625 M, upon which the curve fell, but soon it rose again and, after a further increase of the concentration to 1/500 M, it fell to zero; this latter fall, however, is probably due in part to the approach of metamorphosis, though the rise of the curve soon after the commencement of metamorphosis was due to tetanic convulsions of two larvæ.

As compared with the control thymus series the tetanic convulsions in the Ca lactate series were doubtless somewhat decreased; in particular it is very evident that an increase of the concentration resulted in a fall of the curve. It is worth noting that in the Ca series as in the untreated thymus series each single individual suffered from tetanic convulsions though for a shorter period of its larval life than the larvæ of the thymus-fed, control series.

The most important observation, however, is that though the muscular convulsions were decreased by the action of the Ca salt, the other symptoms of tetany, in particular the permanent paralysis of almost the entire muscular system, developed at the same time and with the same severity as in the untreated thymus-fed control series.

Finally, it should be mentioned that in the Ca series the metamorphosed animals behaved entirely differently from untreated thymus-fed animals. When the larvæ metamorphosed, they were taken out of the solution and placed on moist filter paper. As in all other thymus-fed animals, their muscles were paralyzed and the shape of the body and of the legs was greatly deformed, abnormalities which they had acquired during the tetanic period; like the untreated thymus-fed animals, they did not suffer from tetanic convulsions. Several weeks, however, after metamorphosis tetanic convulsions started again in the Ca animals, in contradiction to what we have observed

in all the untreated thymus-fed animals. This surprising difference has not found so far any explanation and will not be considered in the present article, detailed discussion being reserved until further experiments upon this phenomenon are available.

III. Thymus-Fed Series, Kept in Mg Lactate (Fig. 1, Curve III).— Seven larvæ of *Ambystoma opacum* were used; one of them died soon after the experiment had been started. 8 days after the beginning of the thymus feeding and before tetanic convulsions had made their appearance, the larvæ were transferred to a 1/1250 M solution of Mg lactate in tap water. By this concentration tetanic convulsions were suppressed until the end of the 7th week; at this time one larva had convulsions. But even though the concentration had been lowered to only 1/2500 M, no further convulsions occurred until the 10th week; the concentration was increased to 1/625 M, upon which the curve fell immediately to zero. The concentration was further increased to 1/500 M; only one larva developed tetanic convulsions of the posterior portion of the body, and no further attacks were observed among the larvæ, the rise of the curve at the end of the 18th week being due to tetany of a metamorphosed animal.

In this series the Mg lactate had a distinct and very definite influence upon the frequency and severity of the muscular convulsions; not only is the curve running far lower than in the untreated thymus-fed series, but it is also lower than in the Ca series. Furthermore, three of six larvæ had no muscular convulsions during the larval period. Evidently the effect of the Mg lactate in suppressing the muscular convulsions during tetany is far greater than that of the Ca lactate, when used in the concentrations employed in these experiments.

This influence of the Mg lactate upon the convulsions of the muscles, however, does not mean that Mg is able to suppress tetany, for the other symptoms of tetany, *i.e.* paralysis of the muscles and deformation of the extremities and of the body, develop and to the same degree as in non-treated thymus-fed larvæ.

Concerning the metamorphosed animals the same phenomenon is observed as in the Ca series. A number of weeks after the animals had metamorphosed and were set on moist filter paper without being exposed longer to Mg lactate, they began to suffer again from tetanic

convulsions. And two of the animals which did not have convulsions during their larval period had convulsions after metamorphosis. But the third specimen, which had no convulsions during its larval life, never had tetanic convulsions; it was still alive 43 weeks after metamorphosis.

DISCUSSION AND CONCLUSIONS.

The experiments reported in this article are in full agreement with the facts known about the action of Ca and Mg salts in tetanic animals. In the concentrations used here both Ca lactate and Mg lactate suppressed the muscular convulsions in the tetanic salamander larvæ. The Mg lactate, however, appears to be more effective than the Ca lactate. At any rate the suppression of the tetanic convulsions does not seem to be a specific action of the calcium.

The most important result seems to be the fact that the salts used, though they prevented the muscular convulsions, did not prevent the other symptoms of tetany which in the salamander larvæ are very definite and constant. The permanent spasmodic contractions and the paralysis of the muscles developed in spite of the presence of the Ca and Mg. Furthermore, the muscular contractions and the paralysis developed even in such thymus-fed animals in which the convulsions had been suppressed completely; this was the case in one of the animals of the Mg series.

From the experiments of Biedl[4] and others it is likely that the tetanic convulsions are due to lesions of the central nervous system, since convulsions of a leg can be prevented by isolating it from the central nervous system by cutting the nerves which connect the muscles with the central nervous system. Evidently these lesions of the central nervous system are the chief factor in tetany, while the convulsions of the muscles are only an effect. In the larvæ of salamanders these lesions find a definite expression in the permanent paralysis of almost the entire muscular system.

In the writer's opinion, MacCallum's hypothesis that the tetany toxin has a special affinity for Ca, thereby diminishing the Ca content of the organism, cannot be disproved at present. But the

[4] Biedl, A., Innere Sekretion, Berlin and Vienna, 1913, i, 126.

present experiments seem to prove, first, that the tetany-producing substance causes permanent lesions of the nervous system, which lead to permanent spasmodic contractions and paralysis of the muscle even in the absence of tetanic convulsions, and second, that these cannot be prevented by either Ca or Mg. For the most part they result in an early death of the animals no matter whether or not Ca or Mg has been applied.

In connection with this fact we wish to mention Biedl's claim[4] that no one has yet succeeded in prolonging the life of parathyroidectomized animals by the application of Ca. From MacCallum's paper, on account of the lack of controls, it cannot be seen whether his parathyroidectomized dogs lived longer with Ca treatment than without.

That in spontaneous tetany Ca treatment may effect a cure, as is evident from the report by Howland and Marriott, does not prove that in this case Ca has inhibited tetany as a disease. In spontaneous tetany the period of the action of the tetany-producing substance may be a very short one and the mere prevention of the tetanic convulsions may keep the patient alive until normal function of the glands involved has been restored. The pathological changes which the central nervous system undergoes in this short period may not be severe enough to endanger the life of the patient after the cessation of the action of the tetany toxin.

In the light of the facts presented our experiments lead to the following conclusions:

1. The thymus gland excretes a tetany-producing substance which in the normal animal is antagonized in an unknown way by the parathyroids.

2. In animals devoid of parathyroids (salamander larvæ, parathyroidectomized mammals) this substance may, according to MacCallum, reduce the Ca content of the organism; but by far the most dangerous and important quality of this substance is its highly injurious effect upon the central nervous system, which causes permanent spasmodic contractions of the muscles and paralysis of almost the entire muscular system.

3. It is possible to prevent the muscular contractions by introducing Ca salts into the body, though this can be done more effectively by means of Mg salts.

4. No substance, however, has been found so far to antagonize the tetany toxin and to prevent the development of the lesions of the central nervous system caused by the tetany toxin.

5. This explains why in spite of the application of Ca or Mg and in spite of the suppression by these substances of the tetanic convulsions the other symptoms of tetany develop and frequently lead to the death of the animal.

6. Accordingly the most important function of the parathyroids is to prevent the tetany toxin, by antagonizing it, from coming into contact with the central nervous system.

The writer wishes to express his thanks to Dr. G. M. Meyer, of The Rockefeller Institute, for preparing the solutions used in these experiments.

RATE OF RECOVERY FROM THE ACTION OF FLUORITE RAYS.

BY W. T. BOVIE AND D. M. HUGHES.

(From the Cancer Commission of Harvard University, Boston.)

This paper is a report of some experiments upon the rate of recovery of *Paramecium caudatum* from the cytolytic action of fluorite rays. The organisms were exposed to the radiation emitted through the fluorite window of the hydrogen discharge tube described in previous communications.[1,2] The intensity of the radiation was such that an exposure of 8 seconds caused cytolysis in 57 per cent of the exposed organisms. In order to study the rate of recovery from the action of the rays, the entire 8 seconds of radiation was not given in one exposure, but in two portions of 4 seconds each, with a longer or shorter interval of time intervening between the two exposures. The relation between the length of this interval of time and the percentage of organisms cytolized was observed.

The organisms used were from a pedigreed race of *Paramecium caudatum*, cultured in drops of nutrient infusion on concave microscope slides. A single organism was placed in a small drop (measured to uniform size) and exposed to fluorite radiation on a special microscope slide provided with a fluorite window. The rays passed through the fluorite window of the microscope slide from below. After the exposure the small drop containing the organism was flooded with from one to two drops of infusion and the organism was transferred to a new concave slide and placed in a damp chamber for observation. The following day, the cytolized organisms were disintegrated, while the organisms which survived were active and

---

[1] Bovie, W. T., The action of Schumann rays on living organisms, *Bot. Gaz.,* 1916, lxi, 1.

[2] Hughes, D. M., and Bovie, W. T., The effects of fluorite ultra-violet light on the rate of division of *Paramecium caudatum, J. Med. Research,* 1918, xxxix. 233.

had usually undergone fission. From previous experiments[2] on the effects of these rays on *Paramecium caudatum* we have learned that if the organisms are actively swimming 24 hours after the radiation, they will continue to live and will multiply at the same rate as the non-radiated controls. The experimental results are given in Table I.

It will be seen from Table I that as the interval of time between the two exposures increases, the per cent of cytolized organisms decreases. During the time between the two exposures the organism recovers from the effects of the first 4 second exposure so that when the second 4 second exposure is added the total effect is less than that of a single 8 second exposure. The amount of this recovery in-

TABLE I.

Rate of Recovery from the Action of Fluorite Rays.

Interval of time between exposures.	Total No. of organisms exposed.	No. of organisms cytolized.	Cytolysis.		Calculated No. of organisms cytolized.
				Corrected.	
min.			*per cent*	*per cent*	
0	72	41	57.0	50.0	41.0
7.5	47	20	42.7	35.7	19.75
15	54	17	31.5	24.5	17.0
30	49	9	18.3	11.3	9.35
60	50	3	6.0		4.9

After one exposure of 4 seconds the per cent of cytolysis was 7.

creases as the interval of time between the two exposures increases. For example, when the interval of time between the two exposures was 1 hour, the organism had so completely recovered from the effect of the first exposure that the combined effect of the two exposures was no greater than that of a single 4 second exposure.

7 per cent of all the organisms receiving a single 4 second exposure cytolized. If we subtract 7 per cent from each of the percentages given in Column 4 of Table I, we obtain the values given in Column 5. These values are plotted in Fig. 1 as ordinates against the intervals of time as abscissæ. The points fall upon a smooth curve. The shape of the curve suggested that the process of recovery might be adequately represented by the equations which govern homogeneous
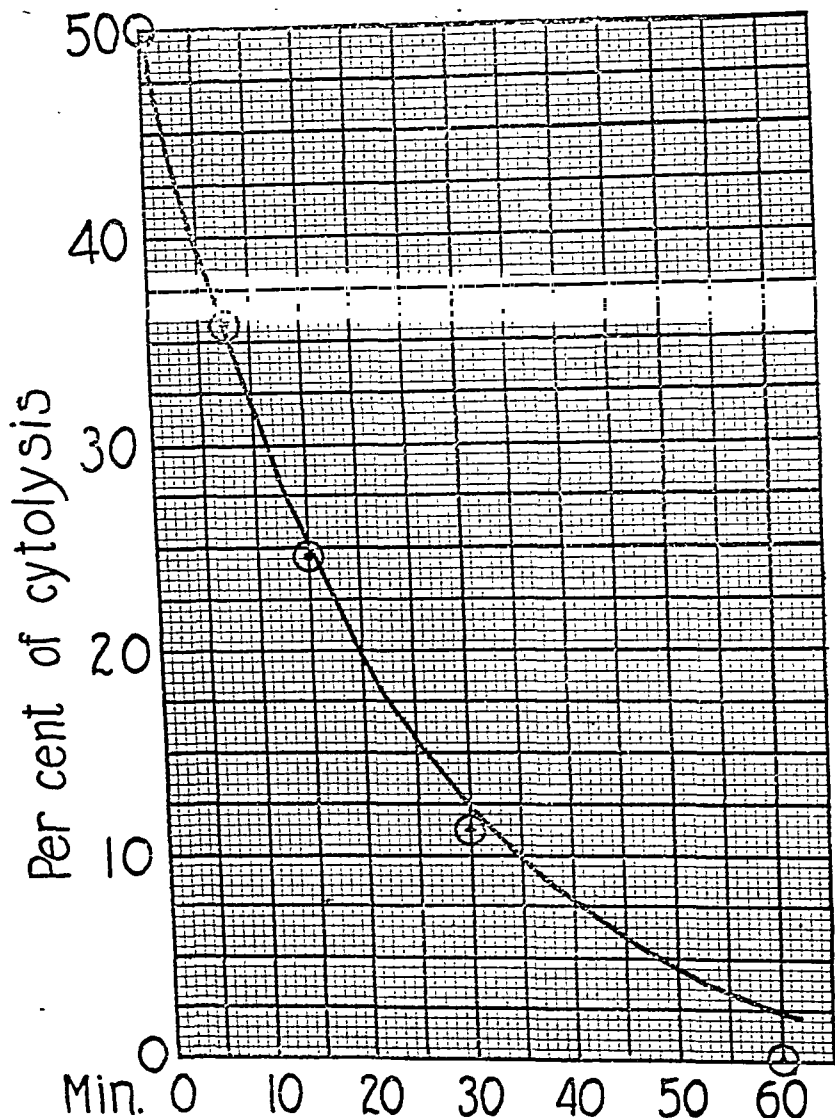
FIG. 1. Graphic representation of the recovery of *Paramecium caudatum* from fluorite radiation as a function of time. The per cents of cytolysis are plotted as ordinates and the intervals of time between the two 4 second exposures are plotted as abscissæ. The curve is a graphic representation of the equation $X = X_0 \epsilon^{-Kt}$, in which K equals 0.0473, X_0 equals the per cent of cytolysis when the time interval is zero, and X the per cent of cytolysis after the intervals of time, t. ϵ is the base of the natural system of logarithms.

mass reactions. I have plotted for comparison the curve for the monomolecular reaction formula

$$X = X_0 \epsilon^{-Kt}$$

when K equals 0.0473, X_0 equals the per cent of cytolysis when the time interval is zero, and X the per cent of cytolysis after the intervals of time, t. The calculated number of cytolized organisms is given in Table I. It will be seen that the observed percentages fall very close to the theoretical curve.

When a number of organisms are exposed to fluorite rays they are not all killed by the same length of exposure, but, owing to individual idiosyncrasies and unknown variations in the experimental conditions,

TABLE II.

Relation between the Frequency of Cytolysis and Length of Exposure to Fluorite Rays.

Length of exposure.	No. of organisms exposed.	No. of organisms cytolized.	Cytolysis.
sec.			*per cent*
6	51	1	2
8	100	29	29
10	106	48	46
12	114	79	69
14	120	109	91
16	105	99	94

some organisms are cytolized by a shorter exposure than others. These differences in susceptibility to the influence of rays may affect the shape of the recovery curve. The nature of the effect will depend entirely upon the relative frequency of cytolysis for various exposure times.

The results of some experiments upon the relation between the length of exposure and the frequency of cytolysis are given in Table II and are represented graphically in Fig. 2. It was not possible in these experiments to duplicate all of the conditions such as light intensity, etc., of the recovery experiments; thus a 4 second exposure did not cause any of the organisms to cytolize. The experimental results are, however, significant in connection with the recovery ex-

periments, for as will be seen by an inspection of Fig 2, for exposures between 6 and 14 seconds duration, with but a single exception,
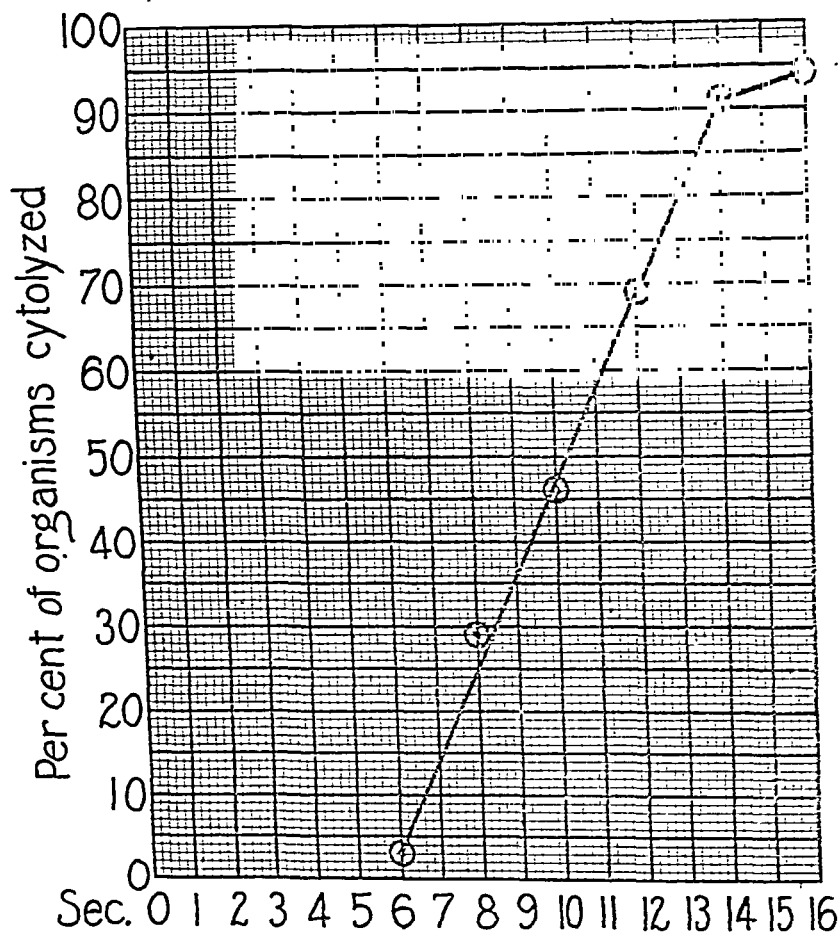

FIG. 2. Graphic representation of the frequency of cytolysis of *Paramecium caudatum* as a function of the length of exposure to fluorite radiation. The per cent of organisms cytolized is plotted as ordinates, the length of exposure as abscissæ.

the curve expressing the relation between the frequency of cytolysis and the length of exposure is practically a straight line. That is, within the limits of experimental error, there are just as many organ-

isms in the class requiring 10 seconds as there are in the classes requiring 12 or 14 seconds exposure to produce cytolysis. The point at 8 seconds is the only one which does not fall upon the straight line and this point could be brought very close to the line by omitting the results of the experiments of 1 day in which the death rate was abnormally high. Such a distribution of susceptibility will have no effect upon the shape of the recovery curve, and we may conclude that the correspondence of the recovery rate with an exponential function of time is not entirely accidental but is the result of an orderly occurrence of the processes involved in the recovery from fluorite radiation.

We may find a cause for such uniformity if we assume that cytolysis occurs when a certain amount of some toxic photoproduct has been formed. (The fact that no cytolysis occurs until the length of exposure is increased to 6 seconds is in harmony with such an assumption.) Recovery then, depends upon the removal of the toxic substance. This removal is accomplished by orderly processes.

Since the rate of recovery is so nearly represented by the monomolecular reaction formula, processes of a chemical nature suggest themselves. Other processes, however, may be conceived. If for example, the toxic substance were removed by diffusion out of the organism, the rate of recovery might correspond very closely to an exponential function of time, especially if the rate of diffusion in the outer limiting membrane were slow as compared with the rate within the cytoplasm. For in this case, since the concentration outside of the organism would, due to the ciliary action, always be zero, the amount of toxic substance diffusing across the membrane in a given time would be proportional to the concentration of the toxic substance remaining within.

Wood and Prime[3] advanced a similar explanation for their observation that it required a much longer exposure to kill carcinoma tissues *in vivo* than *in vitro*. They say[4]: "The constant supply of fresh nutriment to the cells by the blood and the removal of any chemical products formed by the radium in the tissue, must account for this dif-

[3] Wood, F. C., and Prime, F., Jr., The action of radium on transplanted tumors of animals, *Ann. Surgery*, 1915, lxii, 751.

[4] Wood and Prime, *Ann. Surgery*, 1915, lxii, 759.

ference." This suggestion makes the diffusion of toxic substances out of the radiated cell of interest in connection with the toxemias which often follow large doses of either ultra-violet, Roentgen, or γ-radiations.

However, we cannot arrive at very certain conclusions regarding the processes of recovery until these experiments have been repeated with various exposure times, with various radiation intensities, and at various temperatures. Until the data from such experiments are available we may best leave the subject with the simple statement that during the interval of time between the two exposures the organisms recover from the effects of the first 4 second exposure so that when the second 4 second exposure is added the total effect is less than that of a single 8 second exposure. The rate of recovery corresponds very closely to an exponential function of time.

SENSITIZATION TO HEAT DUE TO EXPOSURE TO LIGHT OF SHORT WAVE-LENGTHS.

By W. T. BOVIE AND ALICE KLEIN.

(*From the Cancer Commission of Harvard University, Boston.*)

This paper is a report of some experiments on the effect of heat upon organisms which have been exposed to fluorite rays. The experiments have yielded valuable information concerning the mechanism of the action of rays. They show that the exposed organisms (*Paramecium caudatum*) are made extremely sensitive to the influence of heat.

The experiments are of interest because they show the necessity of a careful control of the temperature of the radiated organisms both during and after the radiation. Heretofore investigators have paid but little, if any, attention to temperature control in their radiation experiments. They have, perhaps, considered the matter unimportant, since the rate of most photochemical reactions is independent of temperature. The effect of heat upon the organism which has been made sensitive to heat by radiation must be clearly distinguished from its effect upon the specific photochemical reaction, for in the former case the effect is upon a series of reactions initiated by the exposure and not upon the photochemical change itself.

The heat sensitization resulting from radiation appears to be of general occurrence. It has been found in *Laminaria* exposed to the rays from radium emanation[1] and in egg white exposed to the rays from a quartz mercury-vapor lamp.[2] In the case of the heat-sensitized egg white visible coagulation occurs at a lower temperature than in the non-radiated control. The degree of sensitization increases with the amount of radiation so that with sufficient expo-

---

[1] Unpublished experiments by the writer.

[2] Bovie, W. T., Temperature coefficient of the coagulation caused by ultraviolet light, *Science*, 1913, xxxvii, 373.

sure coagulation occurs at 0°C. Gelatin, on the other hand, when heat-sensitized by radiation liquefies at a lower temperature than non-radiated gelatin.[3]

The method of experimentation was as follows: By means of a method previously described[4] a single organism from a pedigreed culture of *Paramecium caudatum* was exposed in a small drop of tap water (always of the same size) to the rays emitted through the fluorite window of a hydrogen discharge tube. The drop of water containing the organism was maintained, unless otherwise stated, both before and during the radiation at a temperature of 16–17°C. After the exposure the organism was placed in a larger drop of tap water in the concavity of a microscope slide on the warm plate of an electric stove. The variable junction of a thermocouple (made with No. 30 B. and S. gauge copper and "ideal" wires) was placed in the drop with the organism. The thermocouple circuit contained a galvanometer from which deflections corresponding to 0.1°C. could be read. The temperatures of the dish from which the drop of tap water was taken and the warm plate of the electric stove were so controlled that no change of temperature, capable of being detected by the galvanometer, occurred in the drop containing the organism during the experiment.

No attempt was made to heat all the organisms used in any one experiment to precisely the same temperature. It was sufficient for these experiments to keep the temperature within a certain range. The organisms were exposed to the temperatures indicated below for a period of 60 ± 3 seconds and were then transferred to a culture drop and placed in the damp chamber with the controls for future observation. The experimental results are given below.

Control Experiments.

Experiment 1. Effect of Radiation Alone.—Organisms were radiated at 17–18°C., for 4 seconds and then transferred immediately to culture drops without being placed on a temperature slide.

---

[3] Unpublished experiments by the writer.

[4] Bovie, W. T., and Hughes, D. M., Rate of recovery from the action of fluorite rays, *J. Gen. Physiol.*, 1918–19, i, 323.

```
Total No. of organisms radiated................................  60
No. of deaths..................................................   0
Per cent of death.............................................    0
```

Experiment 1 shows that with the light intensities used, an exposure of 4 seconds to fluorite rays is not sufficient to cause death of the organism.

Experiment 2. Effect of Extra Manipulation of Placing on a Temperature Slide and Then Exposing to Heat.—Organisms were radiated for 4 seconds at 17–18°C., and then exposed on a temperature slide to 16–17°C. for 60 seconds.

```
Total No. of organisms radiated................................  30
No. of deaths..................................................   3
Per cent of death.............................................   10
```

We had observed that some of the radiated organisms appear to be sticky and it was thought that a certain per cent of the deaths in Experiment 5 might be due to accidental physical injury incurred during the transfer to and from the temperature slide. Experiment 2 gives the result of an investigation of this question.

Experiment 3. Effect of Heat Alone.—Unradiated organisms were exposed on a temperature slide to 24–28°C. for 60 seconds.

```
Total No. of organisms........................................  21
No. of deaths..................................................   0
Per cent of death.............................................    0
```

The results obtained in Experiment 3 were to be expected. They are in agreement with those of Woodruff and Baitsell[5] on the temperature coefficient of the rate of reproduction of *Paramecium aurelia*. According to these authors the optimum temperature zone for reproduction is between 24° and 28.5° C.

Experiment 4. Effect of Heating First and Then Radiating.—Organisms were exposed on a temperature slide to 24–26°C.. for 60 seconds, and then radiated for 4 seconds at 17–18°C.

```
Total No. of organisms radiated................................  52
No. of deaths..................................................   4
Per cent of death.............................................    8
```

[5] Woodruff, L. L., and Baitsell, G. A., The temperature coefficient of the rate of reproduction of *Paramecium aurelia, Am. J. Physiol.*, 1911–12, xxix, 147.

Experiment 4 was made in order to determine whether or not we are dealing with an additive effect instead of heat sensitization.

Sensitization Experiment.

Experiment 5. Effect of Radiating First and Then Heating.—Organisms were radiated for 4 seconds at 17–18°C. and then exposed on a temperature slide to 24–26° C. for 60 seconds.

Total No. of organisms radiated.................................. 44
No. of deaths... 29
Per cent of death... 66

Summary.

Experiment No.	Treatment.	Deaths.
		per cent
1	Radiation alone..	0
2	Extra manipulation.......................................	10
3	Heat alone...	0
4	Heating first then radiating.............................	8
5	Radiating first then heating.............................	66

In a previous paper[4] it has been shown that *Paramecia* recover in 1 hour from the effects of a 4 second exposure to fluorite rays, so that an additional 4 second exposure does not increase the effects of the first.

We were interested to see whether the organisms would likewise recover from the heat sensitization caused by an exposure of 4 seconds to fluorite rays. In the following experiments the organisms were not exposed to the higher temperatures immediately after radiation, but in order to permit recovery to take place were kept at the radiation temperature for varying periods of time.

Recovery Experiments.

Experiment 6. Recovery Period ½ Hour.—Organisms were radiated for 4 seconds at 17–18°C., and after ½ hour at 17–18°C. were exposed on a temperature slide to 24–28°C. for 60 seconds.

Total No. of organisms radiated...................................... 20
No. of deaths.. 4
Per cent of death.. 20

Experiment 7.   Recovery Period 1 Hour.—Organisms were radiated for 4 seconds at 17–18°C., and after 1 hour at 17–18°C. were exposed on a temperature slide. to 24–28°C. for 60 seconds.

Total No. of organisms radiated...................................... 21
No. of deaths.. 1
Per cent of death.. 5

Experiment 8.   Recovery Period 5 Hours.—Organisms were radiated for 4 seconds at 17–18°C., and after 5 hours at 17–18°C. were exposed on a temperature slide to 24–28°C. for 60 seconds.

Total No. of organisms radiated...................................... 24
No. of deaths.. 0
Per cent of death.. 0

The organisms had nearly recovered from the heat sensitization caused by the exposure to fluorite rays in 1 hour, and had completely recovered in 5 hours. In this ability to recover the heat-sensitized *Paramecium* differs from the heat-sensitized egg white, since the latter does not recover however long the interval of time between the radiation and the exposure to heat.

The results of these experiments are clear cut, and show that *Paramecia* which have been exposed to fluorite radiation are so sensitized to heat that they are unable to withstand, even for 60 seconds, temperatures which are optimum for non-radiated controls.

Woodruff and Baitsell[5] found normal *Paramecia* from a pedigreed culture able to withstand a temperature of 32° for about 2 days.

In the experiments on egg white, referred to above, it was shown that egg white which is maintained at 0°C., during the exposure to ultra-violet light, will, with the proper amount of exposure. coagulate when it is warmed to room temperature. On the other hand, egg white which is maintained at room temperature while receiving an equal amount of exposure coagulates during the radiation. Now in this case it might not be improper to say that the coagulation is in reality not photocoagulation but is heat coagulation. The egg white has been heat sensitized by the radiation so that it can no

longer withstand normal room temperatures. We might, perhaps, with equal propriety say that the death of *Paramecia* following fluorite radiation is in reality a destruction by heat. The organism, as a result of the exposure, has been sensitized to heat so that it is no longer able to withstand its normal optimum temperatures.

This statement is made in order to emphasize the importance of heat sensitization rather than to suggest an explanation of the nature of the action of rays. Nevertheless, no theory of the action of rays can be complete which does not take the results of such experiments into consideration.

THE PHYSIOLOGICAL BASIS OF MORPHOLOGICAL POLARITY IN REGENERATION. I.

By JACQUES LOEB.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

INTRODUCTION.

In two preceding papers[1] on regeneration in *Bryophyllum calycinum* it had been shown that the mass of shoots formed in a leaf or in a piece of stem to the base of which a leaf is attached increases with the mass of the leaf. This proves that regeneration is a phenomenon of the order of nutrition and growth.

The question arises how this conclusion harmonizes with the well known fact of the polar character of regeneration. When we cut out a piece from a higher plant or a lower animal the piece regenerates, as a rule, a shoot or head at the apical end and roots or a tail at the basal end. In a preliminary paper,[2] published a year ago, the writer described experiments suggesting that the polar character of regeneration might be due to the existence in the circulating sap (or lymph and blood) of inhibitory substances which prevent dormant buds or resting cells from growing out even if an adequate quantity of foodstuffs is available. It was shown by experiments on *Bryophyllum calycinum* that the leaves as well as the growing shoots have an inhibitory influence upon the growth of all the dormant buds situated more basally in the stem. If we assume that these inhibitory influences are due to certain constituents in the sap sent out by growing buds and by leaves, we come to the following theory of the polar character of regeneration. When we cut out a piece of stem from *Bryophyllum* and remove all the leaves, inhibitory substances will continue to flow in a basal direction in the stem. Since the apical

[1] Loeb, J., *Bot. Gaz.*, 1918, lxv, 150; *J. Gen. Physiol.*, 1918, i, 81.
[2] Loeb, *Science*, 1917, xlvi, 547.

region of the piece will be the first to become sufficiently free from
these substances, the buds situated at this end will be the first to
grow out into shoots.   As soon as this happens the new shoots will in
their turn send out inhibitory substances in a basal direction in the
stem, thereby preventing the growth of the more basally situated
buds.   In this paper we shall present some of the qualitative evidence
for the inhibitory effect of a leaf upon shoot formation, leaving the
discussion of experiments of a more quantitative character for a
future paper.
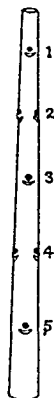
FIG. 1. Diagram showing arrangement of shoot buds in the stem of *Bryo-
phyllum calycinum*.   The line connecting the two buds in one node is always at
right angles to the line connecting two buds in the next node.

We shall deal chiefly with the regeneration of shoots in the stem of
Bryophyllum calycinum, which can proceed only from definitely lo-
cated buds.   In the axil of each leaf of a stem there exists one bud
capable of giving rise to a shoot, which, however, never does so unless
the plant is mutilated.   Each node of a plant has two leaves in
opposite position, and the axis connecting the two axillary buds in
one node is always at right angles with the axis connecting the two
buds of the next node (Fig. 1).   Thus the line connecting the two
buds at Node 2 (Fig. 1) is at right angles with the line connecting the
two buds in Nodes 1 and 3, etc.   The lower leaves in a stem fall off
in time, leaving their axillary buds exposed.   No other element of the
stem except the two buds in each node is capable of growing into shoots.

The elements capable of giving rise to roots are not confined to the nodes but exist all over the stem in a definite layer of the cortex. It can be shown that the sap from the leaf flowing towards the base of a stem favors the growth of roots and inhibits the growth of shoots.

Experiments on Potted Plants.

When we cut off the top of a potted plant of *Bryophyllum calycinum*, leaving a stem containing only two leaves at the apical node. none
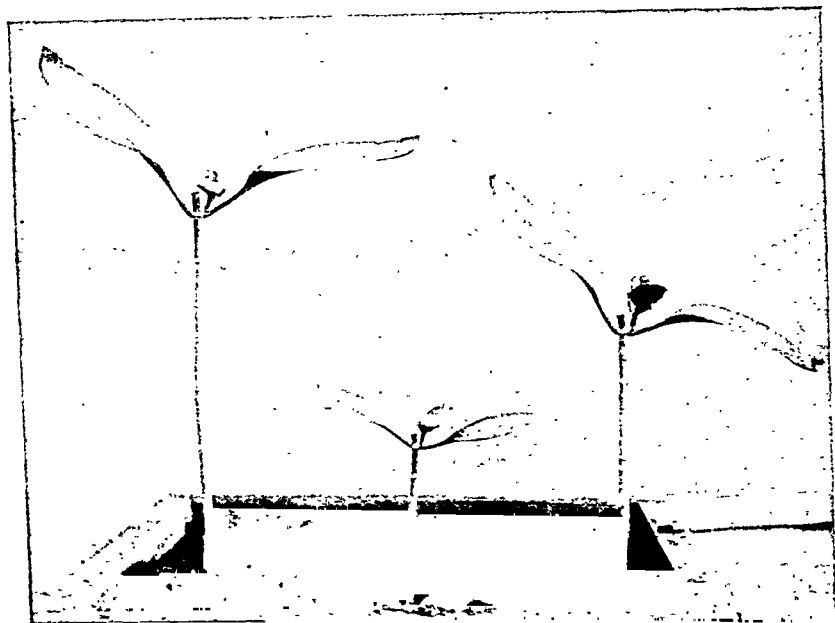
FIG. 2. Top of plant removed; two leaves at apex which prevent growth of all the buds below.

of the buds on the stem below the two leaves will grow out (Fig. 2), so long as the leaves are alive and able to send their sap to the base of the plant. The buds situated in the axil of the two leaves may after some time grow out. The two leaves inhibit therefore the growth of all the buds situated more basally (Fig. 2). Each leaf inhibits the growth of the buds situated in the same half of the stem, and in order to prove this we remove in a second set of experiments

the top of a number of potted plants leaving only one leaf at the apex (Fig. 3).   We must also remove the free bud opposite this leaf, since otherwise this bud will grow out and produce the same inhibitory effect as the removed leaf would have done.   In this case the experiment would only be a repetition of the preceding experiment in which two leaves were left at the apex.   When, however, we remove one apical leaf with its axillary bud very often the one leaf left at the top suffices to suppress regeneration in the basal part of the stem
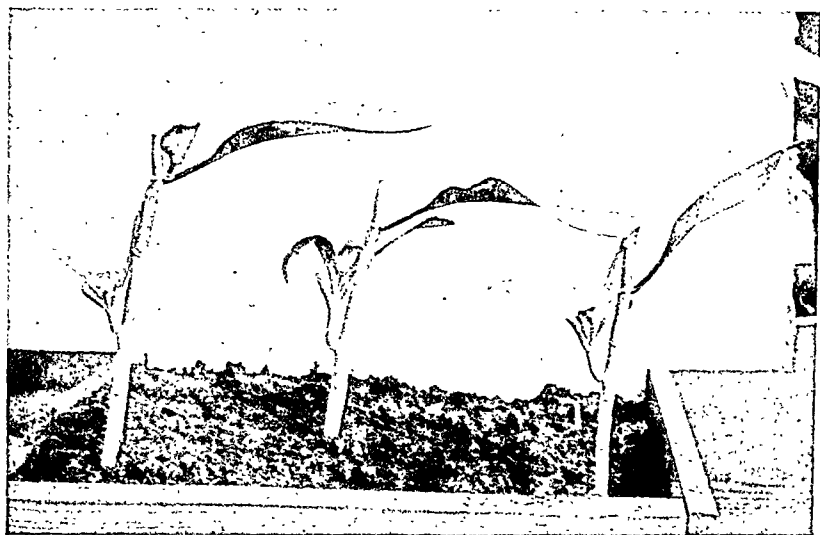
FIG. 3. One leaf left at apex.   Growth of shoots in the first node below the leaf suppressed, while the shoot in the second node below the leaf, but on the opposite side of the stem can grow out.

as completely as if two leaves had been left.   If regeneration occurs, it takes place in the highest node on the opposite half of the stem which is the second node below the leaf (Fig. 3).   In the first node below the leaf no bud can grow out.   I have never noticed an exception to this rule in a normal plant.   A possible explanation of this phenomenon is furnished by Fig. 4, where that half of the stem through which the sap from the apical leaf flows to the base of the stem is shaded.   Since the buds in the first node below

the apical leaf are in the path of the sap flow from the leaf, the formation of shoots is suppressed in these buds, while the bud in the second and fourth nodes below but on the opposite side from the leaf lies outside of the path of the conducting vessels from the leaf. Hence if any bud in such a stem grows out it is usually the one in the second node below but on the opposite side from the apical leaf. As soon as this bud grows out it will inhibit the growth of the lower buds in the same half of the stem.
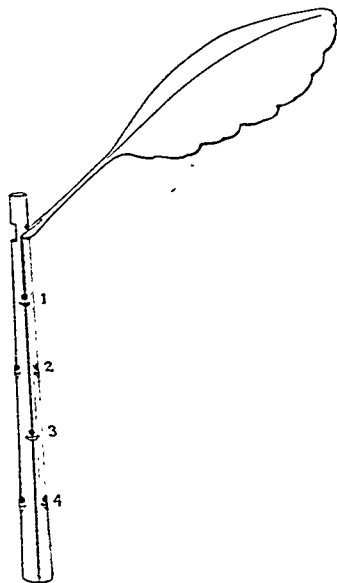
Fig. 4. Diagram explaining this inhibitory influence of the leaf on the theory that the descending sap from the leaf carries inhibitory substances. The region of the stem reached by the sap from the leaf is shaded and in the shaded part regeneration of shoots is inhibited.

The petiole of a leaf is attached with its base to one-half of the circumference of the stem. When we cut off half of the base of the petiole of a leaf, the sap sent out by that leaf can flow only through one quadrant of the next internode. This should limit the inhibitory influence of such a leaf to this quadrant of the node below, and this turns out to be the case. The top of a number of potted plants was cut off and only one leaf was left at the apex (Fig. 5). Half of the
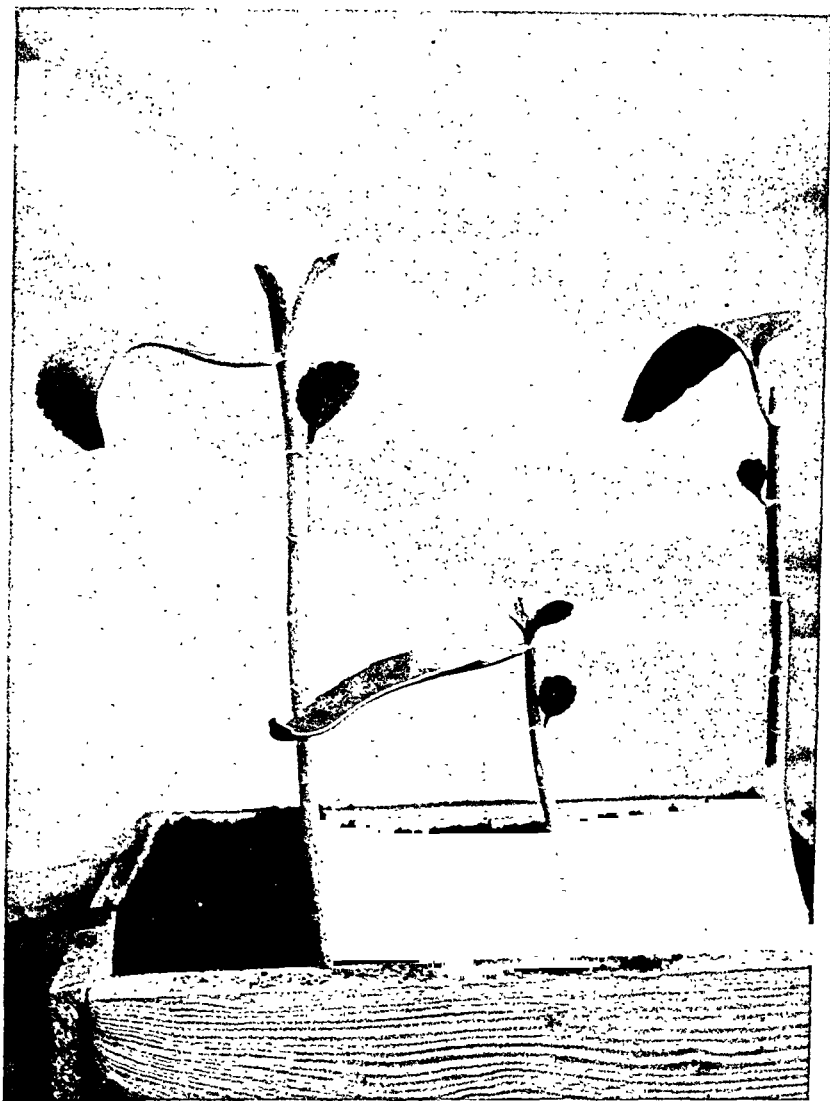
FIG. 5. One-half leaf and one-half petiole left at apex.   One of the two shoots in node below leaf now grows out; namely, on the side where half the petiole is removed.

petiole of this leaf was removed at the base and also the corresponding half of the leaf itself was cut off, though this latter procedure is not essential for the result.   The axillary bud of the other leaf was also cut out as in the preceding experiment.   Fig. 5 gives the result of such an experiment.   The reader will notice that in this case one of the two buds in the first node below the leaf will grow out; namely, that one which lies beneath the removed half of the leaf.   This bud
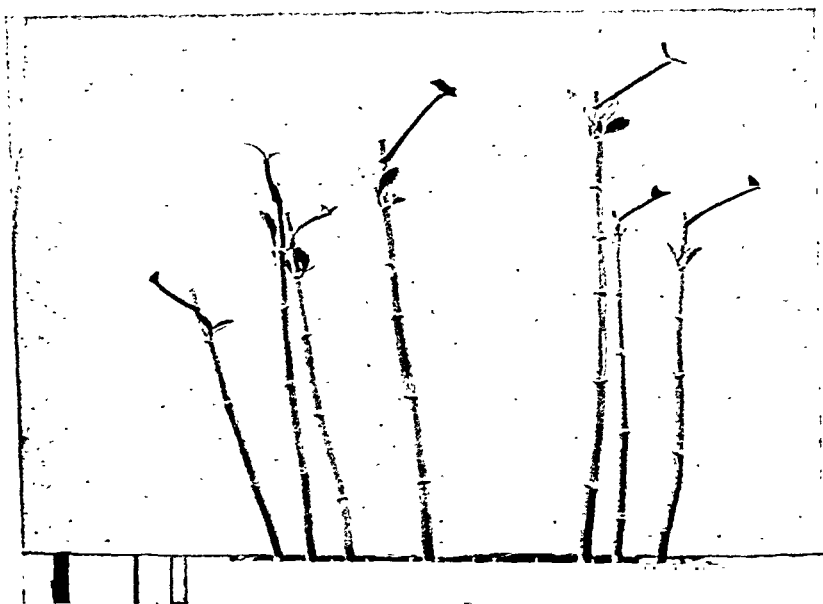
FIG. 6. Showing that the inhibitory influence of an apical leaf upon shoot formation in the node below the leaf disappears when the size of the leaf is sufficiently diminished.   Duration of experiment, Oct. 25 to Nov. 14.

grows out since it no longer receives any of the sap from the leaf above.   I have never seen the other bud in this node grow out.   This experiment also succeeds in practically every case.

We have seen that if we remove one leaf and its axillary bud at the apex of a topped stem, leaving only one leaf at the apex, the buds in the node below are practically always prevented from growing out. When we diminish the mass of the leaf sufficiently (as is done in Fig. 6), this inhibitory influence ceases and every plant forms a shoot in
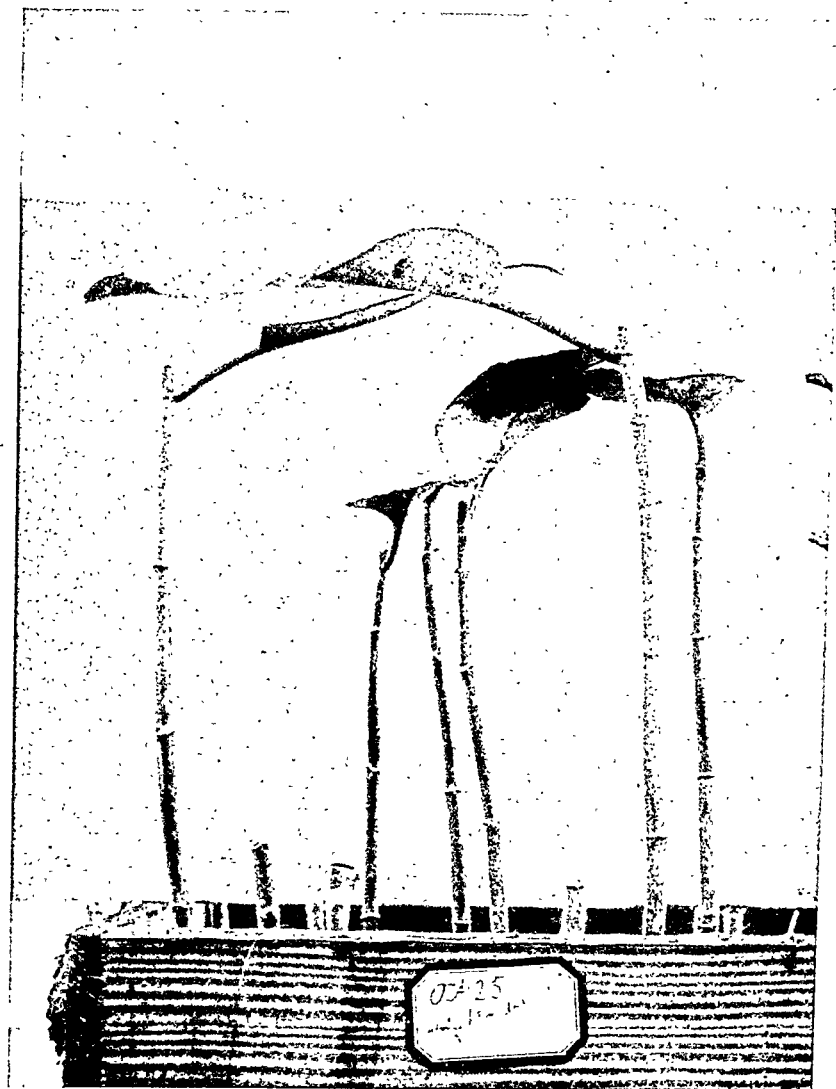
FIG. 7. Control experiment to Fig. 6. Duration of experiment also from Oct. 25 to Nov. 14.

each of the two buds in the first node. Fig. 7 shows the control experiment; namely, six stems each with one whole leaf at the apex. Not a single stem has formed a shoot in the first or any other node below the leaf. Both sets of experiments were carried out simultaneously and both sets of plants were side by side in the same flower bed.

When we reduce the mass of a leaf 10 days after the experiment is started and when the new shoots begin to form, the inhibitory effect nevertheless becomes noticeable.
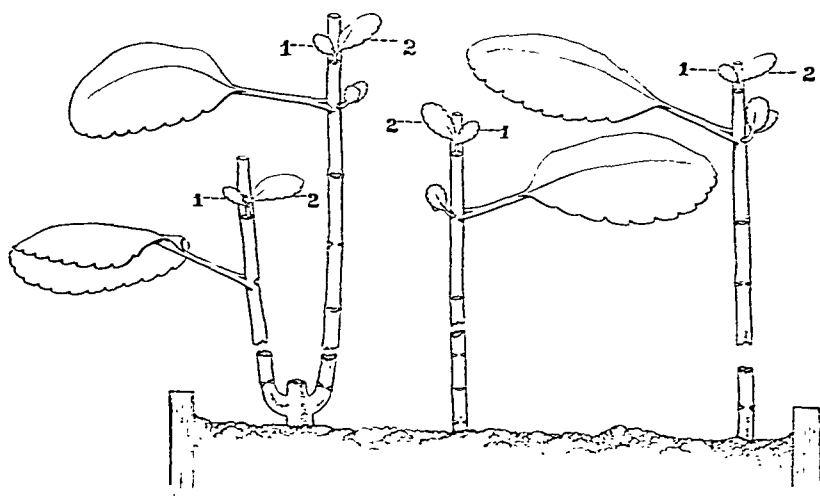
FIG. 8. Proof that traces of inhibition of a leaf upon shoot formation are also noticeable in the more apical shoots. Old leaf left at first node below the apical node. The two Leaves 1 and 2 of the new shoot in the apical node which are normally of equal size show a constant difference, Leaf 1 (on the side where the old leaf is) being smaller than Leaf 2 (on the opposite side).

Demonstration of the Inhibitory Influence of a Leaf on Shoot Formation in a More Apical Node.

We can state as a general rule that a leaf accelerates the growth of shoots at the apex and prevents or retards it in the basal parts of the stem. The leaf has, however, also a slight inhibitory effect on the more apical buds. In order to prove this it is necessary to make experiments like those represented in Fig. 8. In a number of topped

plants one leaf is left at the node below the most apical one.   In this
case both buds in the most apical node grow out into a shoot giving
rise as usual to two small Leaves 1 and 2.   While these leaves are
normally of equal size, a typical and constant difference exists between
the size of the two leaves when one old leaf is left in the node below.
Leaflet 1, which has the same orientation as this old and large leaf in
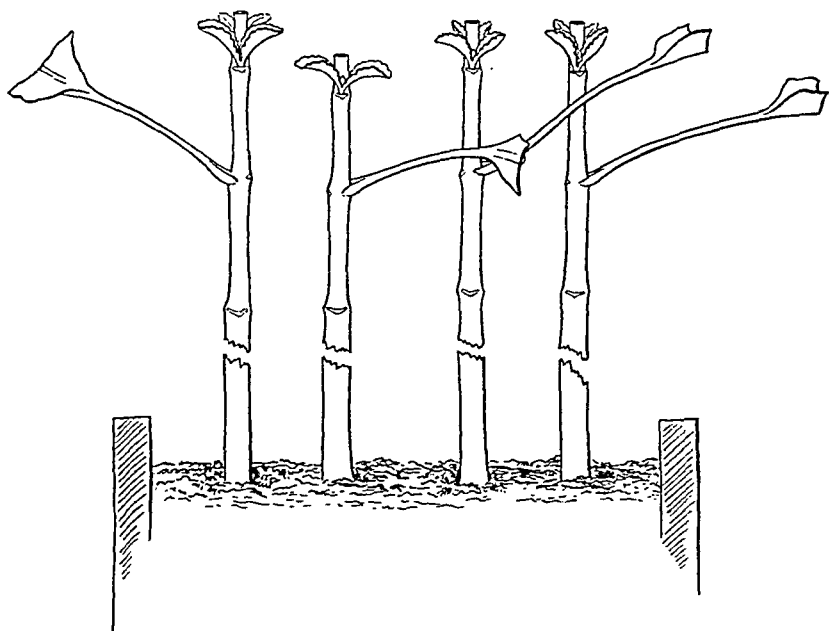
FIG. 9. The inhibitory influence of the leaf upon an apical bud disappears
also when the size of the leaf is reduced.   Duration of experiment, Oct. 24 to
Nov. 7.

the node below, is practically always smaller than the other, Leaflet 2
(Fig. 8).   This difference is intelligible on the assumption that a small
quantity of the inhibitory substances from a leaf flows towards the
apex of the stem; these substances will reach the young leaf facing
the same side of the stem where the old leaf is, while they do not
reach the other leaf.   When we reduce the size of the old leaf, this
inhibitory influence disappears (Fig. 9).
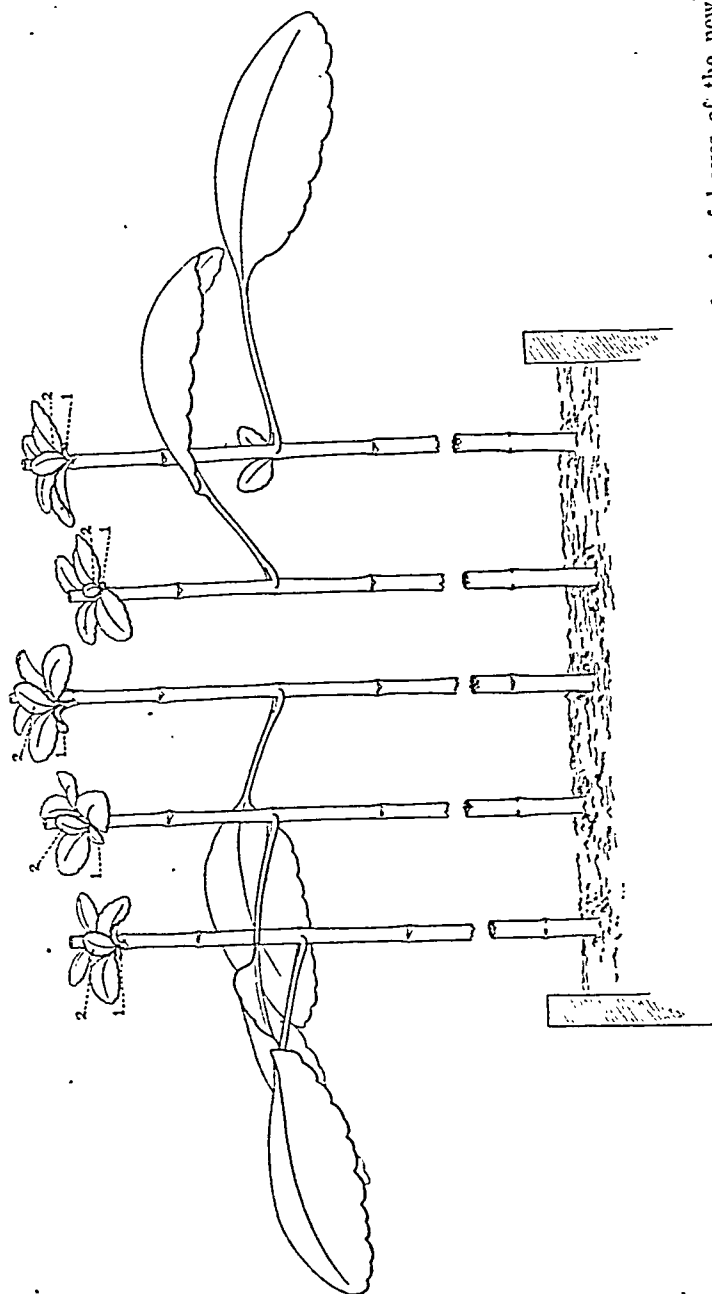
Fig. 10. In the second node above the old leaf the inhibitory effect is shown in the second pair of leaves of the new shoot which are no longer of equal size, Leaf 1 which is above the old leaf being a little smaller than the symmetrical Leaf 2. Duration of experiment, Oct. 17 to Nov. 13.

This slight inhibitory influence of a leaf upon the more apical buds shown in Fig. 8 can also be demonstrated in the growth of the second node above a leaf (Fig. 10).   In this case the influence is noticeable only in the second pair of new leaves of a bud; Leaf 1 which has the same orientation as the old leaf remains smaller than Leaf 2.

While in the basal region of a leaf the inhibitory effect is complete, it is comparatively slight in the apical part.

Influence of Gravity upon the Inhibitory Action of the Leaf.

All the experiments on potted plants described in the preceding pages can be repeated with the same result in stems cut out from a plant. We may omit a description of such experiments since they would constitute only a repetition of what has already been stated. But certain of these experiments yield some additional results which are of theoretical importance.

The assumption that the inhibitory effect of the leaf upon the growth of dormant shoot buds is due to chemical substances sent out by the leaf is supported by the striking influence of gravity on regeneration in stems suspended horizontally.   Long straight stems were cut out from a plant and suspended horizontally in an aquarium nearly saturated with water vapor.   When two leaves are left at the most apical node of such pieces, none of the buds situated more basally will grow out.   If, however, one leaf with its axillary bud is removed and the other leaf left, regeneration will occur, but the buds which will grow out will show a characteristic difference according to whether the leaf is on the upper or the lower side of the horizontally suspended stem.

We suspend such stems so that the axis of the two most apical buds (one of which is removed with its leaf) is vertical (Fig. 11).   In five stems the leaf is on the upper side and in five stems on the lower side of the stem (Fig. 11).   All the stems were originally horizontal but underwent the geotropic bending described in previous papers, whereby the upper side became concave.

When the leaf is below (right half of Fig. 11), shoots may be formed in the first node basally from the leaf.   This occurred in three out of five stems drawn here.   The other two formed shoots from the upper bud of the second node.
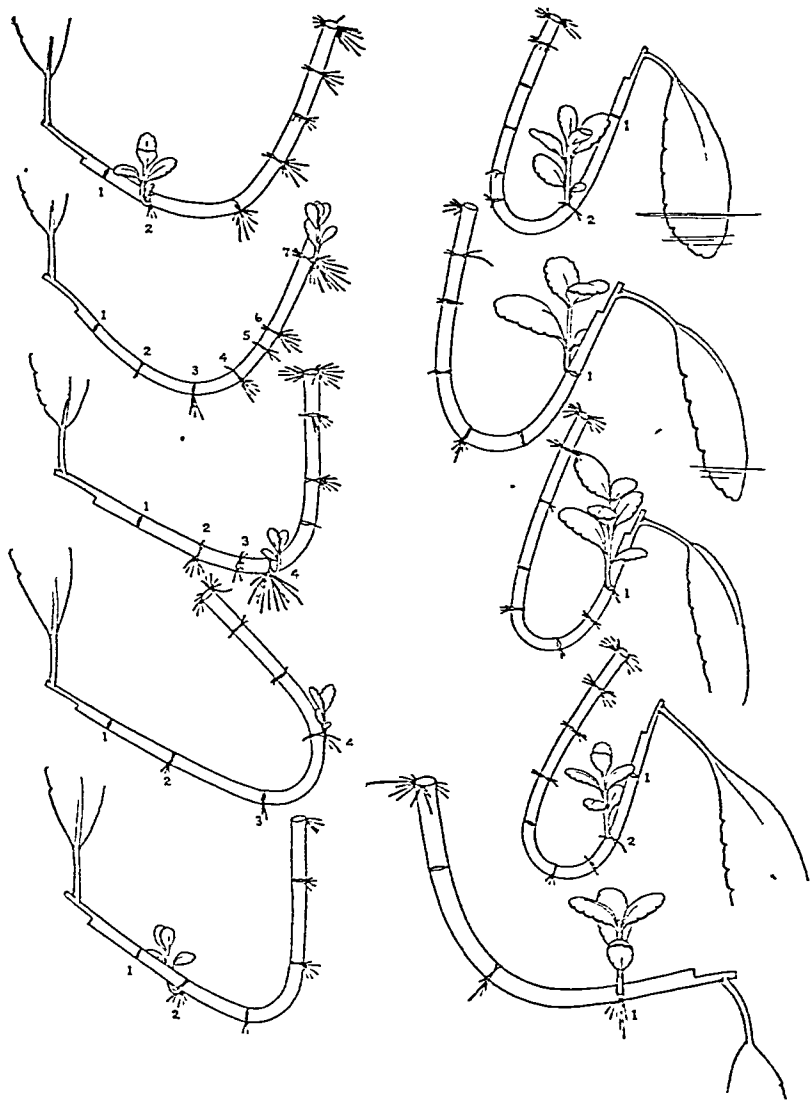
Fig. 11. Influence of gravity on shoot formation in horizontally suspended stems. The stems were originally straight but underwent geotropic bending during the experiment. In the stems to the right one leaf is left at the lower side of apex. In this case the shoots in the first node will grow out in half of the stems; in the second node only the upper bud will grow out. In the stems to the left the leaf is on the upper side. In this case the shoots in the first node are prevented from growing, and only the lower bud in the second and fourth node can grow out. Duration of experiment, Sept. 29 to Nov. 12.

When the leaf is above (left side of Fig. 11) none of the buds in the first node will grow out.   If any buds grow out, they are either the second or fourth on the *lower* side of the stem.   These shoots grow out with much delay compared with the growth of shoots in stems where the apical leaf is on the lower side of the stem.

The inhibitory influence is therefore greater when the apical leaf is on the upper than when it is on the lower side of a horizontally suspended stem.   This influence of gravity supports the idea that it is the sap sent out by the leaf which produces the inhibition.   The diagrammatic Figs. 12 and 13 make this clear.   In Fig. 12 the leaf is below and the path of the conducting vessels from the leaf is marked by black lines.   The two buds of the first node lie on the upper edge of the sap flow containing the hypothetical inhibitory substances   The buds of the first node may or may not receive enough of these substances to prevent their growth.   When, however, the leaf is above (Fig. 13) seepage from the vessels will cause the buds in the first and third nodes to be flooded with the sap and the inhibitory substances contained in it, thus preventing their growth.   The lower bud in Node 2 (or Node 4) is outside the direct path of the conducting vessels of the leaf and hence the lower bud of Node 2 as well as of Node 4 may develop.   Through the influence of gravity traces of the sap may possibly reach the lower bud of the second or fourth node.   This may account for the fact that growth of these buds is usually retarded.

The reader will notice that these facts give us a neat method of restricting the growth of shoots to the lower side of a horizontally suspended stem, contrary to the general rule that in such cases shoots arise on the upper side of the stem.   When we remove the lower half of such a horizontally suspended stem (containing one leaf at the upper side of its apex) leaving on the lower side only the region of the second node (Fig. 14), regeneration of a shoot will occur only from the bud on the under side of this second node.   The growth of the buds in the intact upper half of the stem is completely suppressed and the growth of the bud on the under side of Node 2 is slow for reasons stated.   No growth will occur on the upper side, except after the leaf is wilted or conduction of its sap through the stem is interrupted.

The correctness of this idea is supported by the further fact that this inhibitory effect of a leaf on the growth of shoots, especially in the basal parts of the stem, is diminished when the mass of the leaf is reduced. Fig. 15 shows such an experiment. Of the five stems on the left each had one whole leaf on the upper side of the originally horizontal stem. In one stem only did a shoot form and this shoot
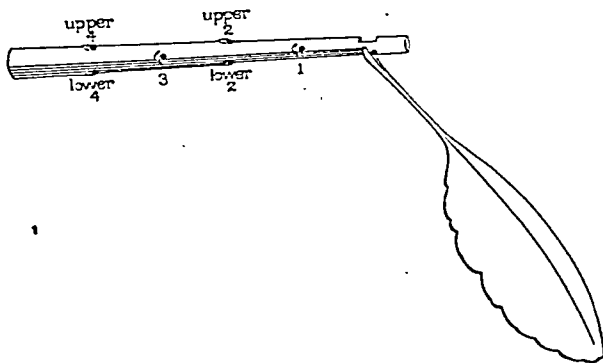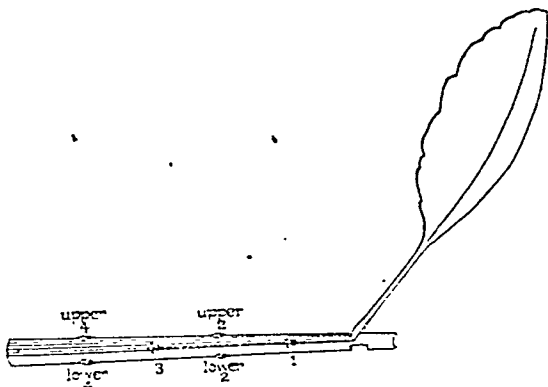

FIG. 12.

FIG. 13.

FIGS. 12 and 13. Explanation of the influence of gravity on regeneration in horizontally suspended stems on the assumption that inhibition is due to substances carried by the sap sent out by the leaf. When the leaf is below (Fig. 12), the buds in Nodes 1 and 3 are at the upper edge of the sap flow and these buds may or may not escape the inhibitory effect. In Fig. 13 the leaf is above and the sap flowing in the upper half is bound to reach the buds in Nodes 1 and 3 and hence their growth is necessarily suppressed. The lower Buds 2 and 4 are outside the sap flow and may develop.

developed on the lower side in the second node. In the five stems on the right the mass of the leaf was reduced considerably. Four of the five stems formed shoots, two even in the second node on the upper side of the stem. In a repetition of this experiment half of the stems with a reduced apical leaf on the upper side formed shoots in the first node basally from the leaf.
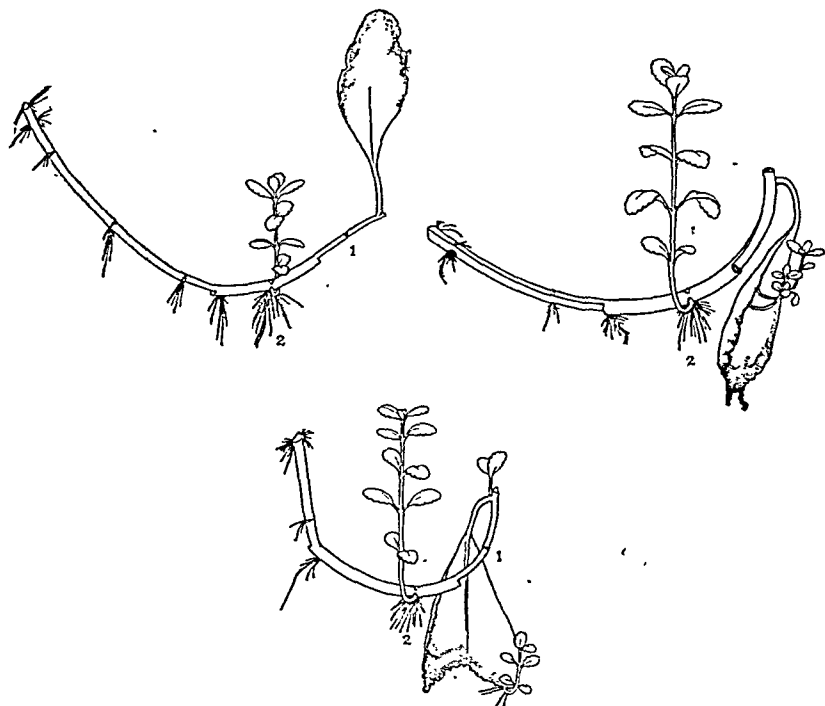

FIG. 14. Horizontally suspended stems. Leaf on upper side. The lower half of stem is removed with exception of second node. The lower bud of the second node grows out into a shoot, while the shoot formation on the upper side of the stem is inhibited by the leaf. Duration of experiment, Feb. 13 to June 24.

The reader will notice that the geotropic bending of the stems was considerably less in the five stems on the right with the reduced leaf than in the five stems on the left with a whole leaf.[3]

[3] Loeb, *Science*, 1917, xlvi, 115; *Ann. Inst. Pasteur*, 1918, xxxii, 1; Forced movements, tropisms, and animal conduct, Monographs on Experimental Biology, Philadelphia, 1918.
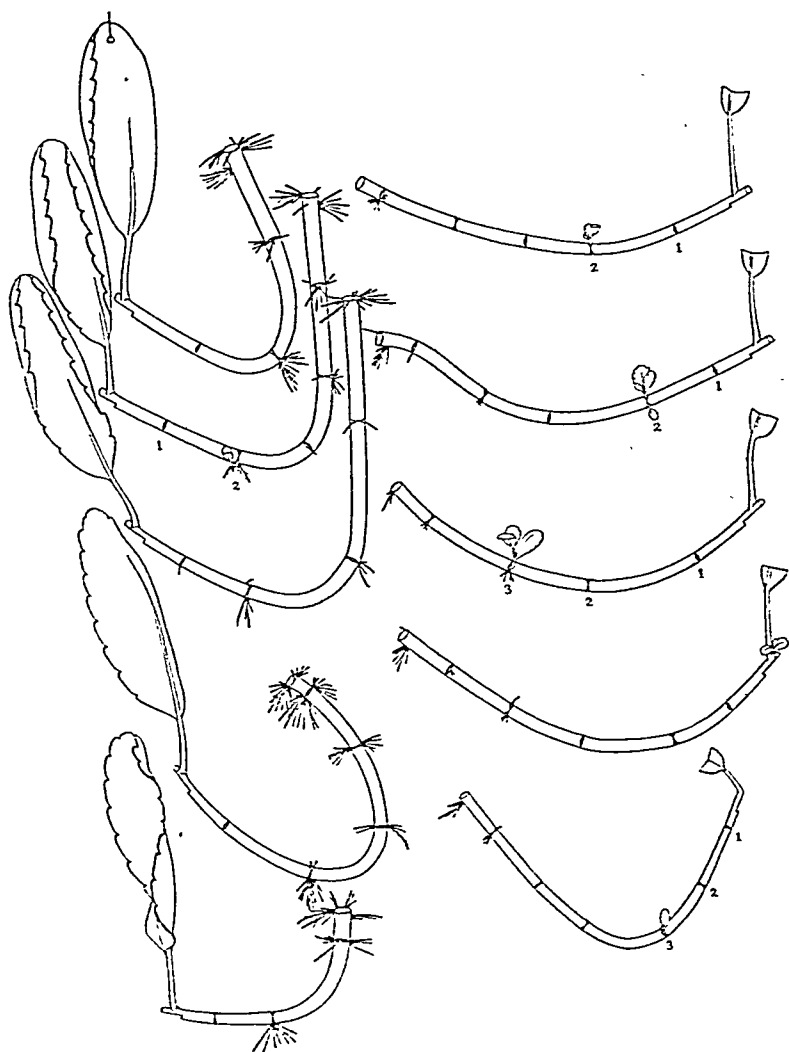
FIG. 15. Showing that reduction in size of apical leaf diminishes its inhibitory power. On the left, five stems each with large size leaf at apex, on upper side of stem. Inhibition of shoot formation complete except in one stem where a shoot is formed in the lower bud of the second node. On the right, five stems each with reduced leaf on the upper side. Four out of five stems form shoots and two of these do so in the upper bud of the second node. Root formation and geotropic curvature are considerably larger in stems with whole leaf than in stems with reduced leaf. Duration of experiment, Oct. 29 to Nov. 23.

Correlation between Inhibitory Effect of a Leaf on Shoot Production and the Opposite Effect on Root Production in a Stem.

The writer has already called attention to the correlation existing between the inhibitory effect of a leaf upon shoot formation and the opposite effect on root formation. This is expressed among others in Fig. 15. The stems on the left side, with a full size leaf at the apex, formed a considerably larger mass of roots in the same time than the stems on the right whose leaves are reduced in size. The larger the apical leaf the greater the mass of roots produced by the basal part of the stem in the same time and under equal conditions; and the greater the inhibitory effect upon the shoot production in this part of the stem.

A striking demonstration of this correlation is given in the upper row of drawings in Fig. 16. One-half of the leaf and one-half of its petiole were cut off. The leaf was at the base of the stem which contained only one node in front of the leaf. Generally only one of the two buds in the node situated apically from the leaf grew into a shoot; namely, the one on that side where half of the leaf was removed. The growth of the bud on the side where the half leaf was preserved was retarded or suppressed. At the basal end of the stems roots developed, but at first only on that side of the stem where the leaf was preserved.[4] Hence the leaf behaves as if it sent out, in addition to the material needed for regeneration, substances retarding shoot formation and favoring root formation.

In the lower row of stems (Fig. 16) the leaf was preserved at the apical end of the stem. In this case the inhibitory effect of the half leaf on shoot formation is much greater than when it is at the base of the stem (upper row). Most of the stems in the lower row have not yet formed any shoots, but where a shoot was formed (as in III*a*) it was formed on the opposite side from that where the half leaf was preserved, while the roots were on the same side with the leaf.

In Fig. 16 the half leaf was above. In Fig. 17 the half leaf was always below. The result in Fig. 17 is the same as in Fig. 16, *i.e.*, on the side where the half leaf is preserved the shoot formation is always retarded compared with that on the other side. The inhibition is more complete when the leaf is at the apex (lower row) than when the leaf is at the base (upper row).

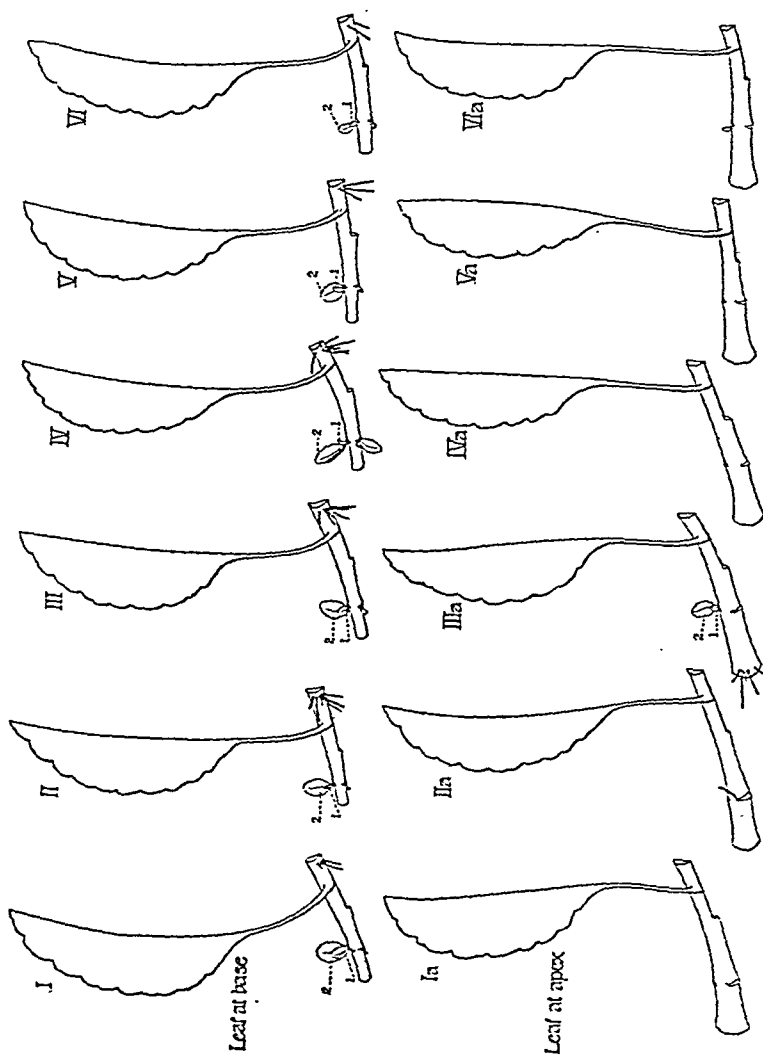[4] Later on roots may form on both sides of the basal end of the stem.

Fig. 16. Upper row: half leaf at base of stem. Shoot formation inhibited and root formation favored on that side of stem where petiole of leaf is preserved. Lower row: half leaf at apex. Inhibitory effect of leaf on shoot formation is more complete than in upper row. Duration of experiment, Oct. 24 to Nov. 9.
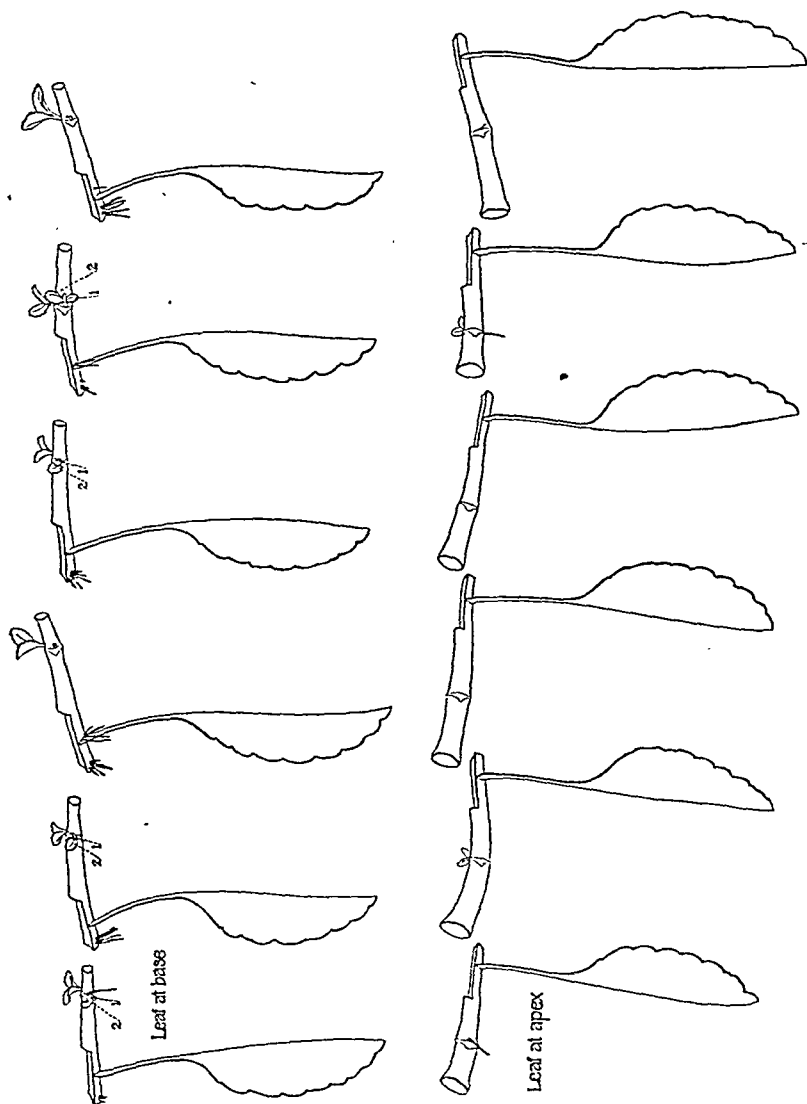
Fig. 17. Half leaf on lower side of stem. Similar result as in Fig. 16. Duration of experiment, Oct. 19 to Nov. 1.

Proof That the Leaf Sends Nutritive Material Also in the Basal Direction of the Stem.

When we suspend stems horizontally with one leaf at the apex, the inhibitory effect of the leaf upon shoot formation is much stronger when the apical leaf is on the upper side of the stem than when the leaf is on the lower side of the stem. We can use experiments of the latter type to show that the leaf sends nutritive substances to the base as well as to the apex and that the fact that the leaf inhibits shoot formation at the base is not due to the leaf failing to send nutritive material in the direction of the base of the stem. The method of proving this consists in measuring the influence of the mass of the leaf upon shoot formation in the basal part of a horizontally suspended stem.

Stems were split longitudinally and suspended horizontally; each stem having a leaf at the apex, and on the lower side of the stem.[5] Fig. 18 gives the result of such an experiment. Pieces of stems possessing two nodes and two leaves at the apical node are split longitudinally, so that each half stem has one leaf at the apex and one bud in the basal node. One leaf is left intact while the size of the sister leaf is reduced considerably. In Fig. 18 Leaves I and Ia, II and IIa, etc., are sister leaves. Practically each stem has produced a shoot at the basal node, but the shoot is invariably greater in the stems in the upper row where a whole leaf was at the apex than in the lower row where the apical leaf was reduced in size. The drawing was made on the 34th day of the experiment. It is obvious that the growth of the basal shoots increases with the mass of the apical leaves and this is proved by the relative weight of the leaves and shoots.

Duration of Experiment, 39 Days.

	Wt. of 4 whole leaves.	Mass of 4 shoots.	Shoots per gm. of leaf.
	gm.	*gm.*	*ns.*
Fresh.	17.035	2.128	125
Dry.	1.196	0.193	160
	Wt. of 4 reduced sister leaves.		
Fresh.	2.182	0.384	175
Dry.	0.140	0.027	193

[5] If the leaf is on the upper side, we get too much inhibition of shoot formation, whereby the experiment is rendered difficult.
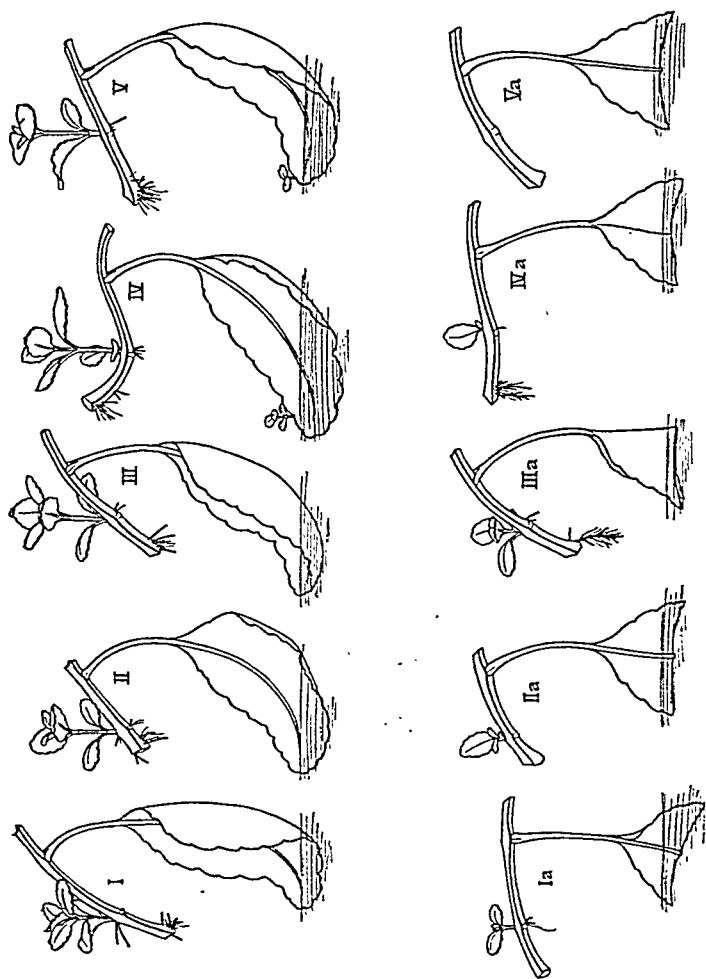
Fig. 18. Stems split longitudinally and suspended horizontally, with apical leaf on the lower side. Leaves I and Ia, II and IIa, etc., are sister leaves. The leaf in the upper row is intact, while in the lower row it is reduced in size. The mass of shoots produced is smaller in the stems of the lower row where the size of the leaf is reduced than in the upper row where the leaf is larger. This proves that inhibitory influence of leaf upon basal parts of stem is not due to lack of nutritive material.

Other experiments gave similar results. While the experiment does not reveal any strict proportionality between mass of leaf and mass of basal shoot produced, the increase of the mass of shoots with the mass of the leaves is unmistakable.

We therefore come to the following conclusion. The leaf sends material for growth in both directions of the stem, to the apex as well as to the base. It also behaves as if it sent out inhibitory substances in both directions, but if this be the case there must exist a considerable difference in regard to the mass of the latter. While much or almost all of the inhibitory substance is sent in basal direction, only traces of it are sent in an apical direction, so that special experiments are required to demonstrate the inhibitory effect in the apical parts of the stem.

A glance at Fig. 18 confirms also the statement that the mass of roots formed in the basal part of a stem increases with the mass of the apical leaf. The stems in the upper row with a whole leaf at the apex have a greater mass of shoots and roots than the stems in the lower row with a reduced leaf.

DISCUSSION.

We have shown in this paper that a leaf inhibits the regeneration of shoots in the basal parts of the stem and that this inhibition is diminished or ceases when the mass of the leaf is reduced below a certain limit. If the inhibitory influence of the leaf is due to inhibitory substances sent out by the leaf to the basal parts of the stem we must conclude that traces of these inhibitory substances flow also to the apex of the leaf since it is possible to demonstrate slight inhibitory influences of the leaf in the buds situated apically.

The influence of the leaf upon the regeneration of roots is exactly the reverse from that on the regeneration of shoots. The leaf favors the formation of roots in the basal parts of the stem and this favorable influence upon regeneration of roots in the basal part of a stem increases with the mass of the apical leaf.

This gives us an indication of the rôle which a leaf plays in the establishment of the polar character of regeneration in the stem of *Bryophyllum calycinum*. When a piece of stem is cut out with a leaf in the middle, the leaf sends out nutritive material in both directions of

the stem, since it can be shown that if once a shoot is caused to grow, it increases with the mass of the leaf, no matter whether the shoot is situated at the base or the apex.   The leaf has a powerful inhibitory effect upon the development of basal shoots.   If we assume this inhibitory influence to be due to inhibitory substances we must further assume that not more than mere traces of these inhibitory substances reach the apex which are not sufficient to interfere with the growth of shoots. At the moment we cut out the piece of stem from a plant the stem contains throughout a sufficient quantity of these inhibitory substances to prevent shoots from growing, and these inhibitory substances will continue to flow in the descending sap towards the base of the stem. The most apical buds in the stem will hence be the first ones to become sufficiently free from inhibitory substances to be able to grow and the regeneration of shoots will start at the apex of the piece of stem. As soon as the shoots are beginning to grow at the apex they in turn act like a leaf so that now the further growth of shoots at the base is permanently inhibited.   On the other hand, the influences which inhibit shoot formation at the base are associated or identical with influences favoring root production.   Hence the leaf will favor root formation at the base of the stem and shoot formation at the apex. This gives an idea how the leaf may contribute by its "internal secretion" to the establishment of the polar character of regeneration.

If it could be shown that plants possess a closed circulatory system comparable to that of animals, all these facts might become easily intelligible if we assume that inhibitory substances for shoot formation (and favorable substances for root formation) are carried in the descending sap from the leaf to the root, where they are retained or altered, so that the ascending sap becomes practically (but not absolutely) free from these substances and contains only the nutritive material for the formation of shoots.

The assumption that the inhibitory influence of a leaf upon shoot formation in the basal part of a stem is due to inhibitory substances, is not without analogy in biology.   It is known that when twins in cattle have different sex the female is in the majority of cases sterile, and Lillie[6] has shown that there exists an exchange of blood between

[6] Lillie, F. R., *J. Exp. Zool.*, 1917, xxiii, 371.

such embryos. This indicates that there exists in the blood of the male cattle embryo an inhibitory substance which prevents the normal development of the sex glands of the female embryo.

A second case is that of the prevention of the development of the male plumage in the female fowl. Boring and Pearl[7] have shown that the ovary of such females contains specific cells, the lutear cells, which are absent in the male. Boring and Morgan[8] have found that in the Sebright, where the male shows hen-feathering, lutear cells exist in the testes of the male bird. Since extirpation of the ovary in fowl and duck leads to the assumption of the full male plumage by the female (Goodale[9]), it seems as if some specific substance in the ovary inhibited the development of male plumage in the female. This inhibitory substance may be contained in the lutear cells, which, however, cannot well influence the development of feathers in any other way than by the secretion of some substance into the blood. The assumption that the inhibition of shoot formation in the basal part of a stem by a leaf is due to an inhibitory substance secreted by the leaf is therefore not without a precedent.

It is, however, necessary to call attention to the fact that even if the inhibitory influence of the leaf upon shoot formation should turn out to be based on the chemical character of the sap sent out by the leaf, it does not follow that all phenomena of inhibition and correlation in regeneration will find their explanation on the same basis. Quantitative experiments published in a former paper suggest that the inhibitory influence of a piece of stem on shoot formation in the leaf of *Bryophyllum calycinum* is due to the fact that the leaf sends its sap normally to the stem, and that as long as this happens the buds in the notches of the leaf cannot grow out.[10]

We shall show in the next communication that growing buds have inhibitory influences upon the formation of shoots comparable to the same influences caused by a leaf.

[7] Boring, A. M., and Pearl, R., *Anat. Rec.*, 1917, xiii, 253. Pearl and Boring, *Am. J. Anat.*, 1918, xxiii, 1.

[8] Boring, A. M., and Morgan, T. H., *J. Gen. Physiol.*, 1918, i, 127.

[9] Goodale, H. D., *Biol. Bull.*, 1910–11, xx, 35; *Am. Nat.*, 1913, xlvii, 159; *J. Exp. Zool.*, 1916, xx, 421.

[10] Loeb. *Ann. Inst. Pasteur*, 1918, xxxii, 1.

SUMMARY.

1. In *Bryophyllum calycinum* two apical leaves suppress the shoot formation in all the dormant buds situated basally from the leaf; one apical leaf suppresses the shoot formation in the basal buds situated in the same half of the stem where the leaf is, and, if one-half of the petiole of such a leaf is removed, the growth of basal buds in one quadrant of the stem is suppressed.

2. This inhibitory influence of a leaf upon shoot formation in the basal part of a stem is diminished or disappears when the mass of the leaf is reduced below a certain limit.

3. The inhibitory influence of an apical leaf upon the growth of shoots in horizontally suspended stems is greater when the leaf is on the upper than when it is on the lower side of the stem.

4. All these facts suggest the possibility that the inhibitory influence of the leaf upon shoot formation is due to inhibitory substances secreted by the leaf and carried by the sap from the leaf towards the base of the stem.

5. An apical leaf accelerates root formation in the basal part of a stem and this accelerating effect increases with the mass of the leaf.

6. This inhibitory influence of a leaf upon shoot formation and the favoring influence upon root formation in the more basally situated parts of the stem is one of the factors determining the polar character of regeneration.

AMPHOTERIC COLLOIDS.

III. CHEMICAL BASIS OF THE INFLUENCE OF ACID UPON THE PHYSICAL PROPERTIES OF GELATIN.

BY JACQUES LOEB.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

I.

, Many of the authors who have worked on the physical chemistry of proteins, like Hardy, Pauli, Michaelis, Robertson,[1] and others, have pointed out that the different properties of proteins, *e.g.* swelling, viscosity, are affected by electrolytes in a parallel way, a fact which suggests that these variations are due to the same variable. The nature of this variable is not known and the majority of authors believe it to be connected with the colloidal character of the proteins, while others are inclined to assume a purely chemical or stoichiometrical relation. The reason for this doubt lies in the fact stated appropriately by Pauli[2] in discussing the influence of acid and alkali upon the osmotic pressure of gelatin.

Pauli and Handowski have pointed out that in these experiments too the essential feature is the formation of ionic protein. But a satisfactory explanation of this increase is still lacking, because we have no measurements of the molecular concentrations with the aid of other methods, which prove that we are dealing with a true osmotic pressure in the sense of van't Hoff.

Pauli assumes that the ionized protein undergoes a stronger "hydratation" than non-ionized protein and that this hydratation explains the swelling of gelatin, as well as the apparent osmotic pressure, the latter being only a phenomenon similar to swelling.

[1] The reader is referred for the literature on the subject to Robertson, T. B., The physical chemistry of the proteins, New York, 1918.

[2] Pauli, W., *Fortschr. naturwiss. Forschung*, 1912, iv, 245.

It seemed to the writer that experiments on gelatin might permit us to satisfy the demand of Pauli; namely, to supply the molecular measurements necessary to show that the osmotic pressure and the other properties of gelatin solutions vary in proportion to the amount of acid combining with a given amount of gelatin. In two previous papers the writer has already shown that this is true for the influence of neutral salts on these properties of gelatin.[3] Procter's[4] experiments also indicate a purely stoichiometric basis for the influence of acids on the swelling of gelatin.

According to Werner[5] amphoteric electrolytes are characterized by their ability to add H ions or OH ions and not by their ability to give off H and OH ions. It is generally assumed, and probably correctly, that when an acid like HBr combines with an amino-acid or a protein, the reaction occurs in an NH_2 group of the amino-acid or protein. According to Werner when NH_3 and HBr combine, the positively charged hydrogen ion of HBr is attached by a secondary valency to the N whose three negative charges now hold four positively charged H ions. No other positive ion except H can act in this way. The Br is able to dissociate as freely in the NH_4Br as in the free acid. The same assumption is to be made for the way an acid, e.g. HBr, combines with amino-acids or proteins.
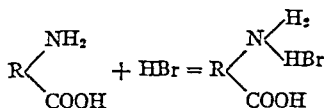
Such a molecule, e.g. gelatin bromide, dissociates into a positively charged gelatin ion and a negatively charged Br ion, the H ion of the HBr now forming a part of the complex and positively charged gelatin ion.
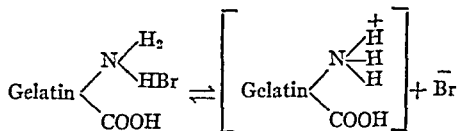
[3] Loeb, J., J. Gen. Physiol., 1918, i, 39, 237.

[4] Procter, H. R., and Wilson, J. A., J. Chem. Soc., 1916, cix, 307. Procter, H. R., and Burton, D., J. Soc. Chem. Ind., 1916, xxxv.

[5] Werner, A., Neuere Anschauungen auf dem Gebiete der anorganischen Chemie, Braunschweig, 2nd edition, 1909.

Such a gelatin salt can only exchange the Br with the anion of a neutral salt and it is impossible for a complete molecule of a neutral salt like KCl to combine with the NH_2 group as has been assumed. The writer's experiments on the action of neutral salts on gelatin treated previously with acid are in harmony with the ideas of Werner and opposed to the assumption of a pentavalent N atom in the protein molecule capable of adding a whole molecule of a neutral salt. We do not know yet whether only one or more NH_2 groups in the gelatin molecule are able to bind a molecule of HBr.

Gelatin is an amphoteric electrolyte which at the isoelectric point (which for gelatin lies at pH = 4.7) is practically insoluble. When we prepare a gelatin solution and give it a hydrogen ion concentration of 2.10^{-5} (*i.e.* pH = 4.7), the solution in less than 24 hours becomes opaque on cooling in as low a concentration as 0.25 per cent and probably at any concentration; except that the opacity due to the insolubility becomes too slight in very low concentrations to be noticeable. This explains why gelatin at the isoelectric point has practically no osmotic pressure, no swelling, a minimal conductivity, viscosity, etc.

When we treat isoelectric gelatin with a limited quantity of HBr of a low concentration a certain amount of gelatin is transformed into gelatin bromide, which is soluble and dissociates electrolytically. The higher the concentration of acid used the more gelatin bromide is formed and the more molecules of gelatin go into solution, until at a certain point all the insoluble gelatin molecules are converted into soluble gelatin bromide molecules. Since a 1 per cent gelatin bromide solution should possess the same degree of electrolytic dissociation as the HBr combined with it and since a 1 per cent gelatin bromide solution on account of the high molecular weight of gelatin must be considered as a very dilute solution, we shall commit no great error in assuming a complete electrolytic dissociation of the gelatin bromide. If it is true that the increase in osmotic pressure of gelatin under the influence of HBr is merely due to an increase in the number of soluble gelatin molecules, it must be possible to show that the osmotic pressure in this case increases approximately with the number of gelatin bromide molecules formed. This we intend to prove in the present paper.

While Pauli is right in stating that gelatin treated by acid is more highly ionized than common gelatin, I cannot agree with him that it is this ionization which causes the difference in the osmotic pressure of the gelatin at the isoelectric point and gelatin treated with acid. The increase in osmotic pressure is as our numerical results will show merely the expression of the increase in the number of particles in solution and there is no need or room for the assumption that the hydratation or any other quality except the number of particles in solution has anything to do with this increase in pressure.

Since viscosity and swelling vary practically parallel with the osmotic pressure, these phenomena must also be a function of the number of particles or ions in solution.

Hardy[6] has pointed out that the ionization of a protein increases its viscosity, and the increase of viscosity of gelatin with its ionization might then account for the parallelism between the curves for the bromine number and for the viscosity of the gelatin solution.

As far as a theory of swelling is concerned, the only one possessing any quantitative basis at present is Procter's.[4]

If we can, therefore, prove that under the influence of HBr the osmotic pressure of gelatin changes in proportion with the gelatin bromide formed, we have no further reason to question the purely chemical or stoichiometrical basis of the influence of acid upon all the physical properties of gelatin.

II. Measurements without Washing.

1 gm. of finely powdered gelatin is put for 30 minutes at 15°C. into each of a series of beakers containing 100 cc. of HBr of a different concentration, varying from M/8 to M/8192. As a control 1 gm. of gelatin is put for 30 minutes at 15°C. into 100 cc. of distilled water. The powdered gelatin is then poured into a cylindrical funnel and the acid allowed to drain off. The diameter of all the funnels being the same, the height of the gelatin gives a measure for the relative amount of swelling. Each gram of gelatin is then put into 100 cc. of HBr of the same concentration with which it had been treated before and is liquefied by heating to 50°C. Immediately after melting the time

6 Hardy, W. B., *J. Physiol.*, 1905–06, xxxiii, 251.

of outflow through an Ostwald viscometer is ascertained at a temperature of 24°C. By keeping temperature and time required for melting and time between completing this process and the viscosity measurement constant in each case comparable results are obtained.[7] The time of outflow of distilled water through the viscometer was 55 seconds. The two curves of Fig. 1 give the values for swelling and viscosity, with the logarithms of the concentration of acid used as abscissæ. Under each acid is found the pH for the gelatin solution ascertained after the viscosity determination.
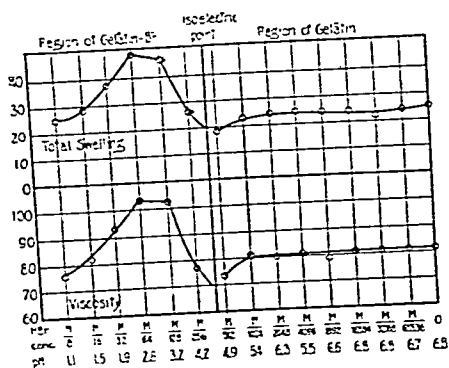

FIG. 1. Curves for viscosity and swelling of gelatin in the presence of various concentrations of HBr. Abscissæ, logarithms of concentration of HBr used. Under each concentration is found the pH of the solution. Isoelectric point and minimal values for curves at pH about 4.7. Both curves parallel, showing a maximum at pH between 2.8 and 3.2, and a drop when pH < 2.8.

The rest of the liquefied solution of gelatin in acid was then put into bags of collodion,[8] to ascertain the osmotic pressure of the gelatin solution, the pressure being expressed in mm. of height of the column of 1 per cent gelatin solution in the glass tube inserted through the tightly fitting rubber stopper closing the bag of collodion. The

[7] Loeb, J. Biol. Chem., 1918, xxxiv, 395.

[8] Lillie, R. S., Am. J. Physiol., 1907–08, xx, 127. Loeb, J. Biol. Chem., 1918, xxxv, 497. It is hardly necessary to state that these bags are freely permeable for HBr and that HBr produces no osmotic pressure when put in such bags. There is a slight rise of the column of liquid in the manometer at the beginning of the experiment which disappears completely in a few hours, while the rise due to the gelatin, for which the membrane is impermeable, is permanent.

outside liquid was in each case HBr of the same concentration as that
in which the gelatin was dissolved.   The whole experiment was car-
ried on in a water bath of a temperature of 24°C.   The curve at the
top in Fig. 2 is the curve for the osmotic pressure observed after
about 20 hours, at which time equilibrium was established.

The conductivity of the gelatin solution was then ascertained, as
well as the amount of Br found in 25 cc. of 1 per cent gelatin solution.
The curves are given in Fig. 2.   A glance at Fig. 2 will show that the
curve for osmotic pressure is *not* parallel to the curves for conductivity
and for the Br number.   It is, therefore, impossible to arrive from
experiments of this type at a decision whether the influence of HBr
(or other electrolytes) upon gelatin is of a stoichiometrical or of a col-
loidal character.   Yet those familiar with the literature of this sub-
ject will remember that the conclusions of most colloid chemists are
based on experiments in which the action of the electrolyte upon the
protein was measured in the presence of an excess of electrolyte.
The second fact which deserves attention becomes clear by a com-
parison of Figs. 1 and 2; namely, that the minima which appear in
the two sets of curves lie at different acid concentrations: in Fig. 1
between M/256 and M/512, in Fig. 2 (for osmotic pressure) between
M/2048 and M/4096.   Comparing, however, the pH in the two sets of
curves we notice that the minimum is at the same pH, namely about
4.7, which is the isoelectric point for gelatin.   A good deal of the work
on which the colloidal theory of the behavior of proteins rests was
done without any measurements of pH and by plotting the effect
against the concentration of the acid.   It is no wonder that work
with two such shortcomings in its method did not furnish any proof
for the stoichiometrical character of the action of electrolytes on the
physical properties of amphoteric colloids.

III. Effect of Washing.

Our method consists in removing the excess of HBr (or of any other
electrolyte) after it has had a chance to act on the gelatin.   1 gm. of
powdered gelatin is put again into each of a series of beakers for 30
minutes at about 15° or 20°C., each beaker containing 100 cc. of
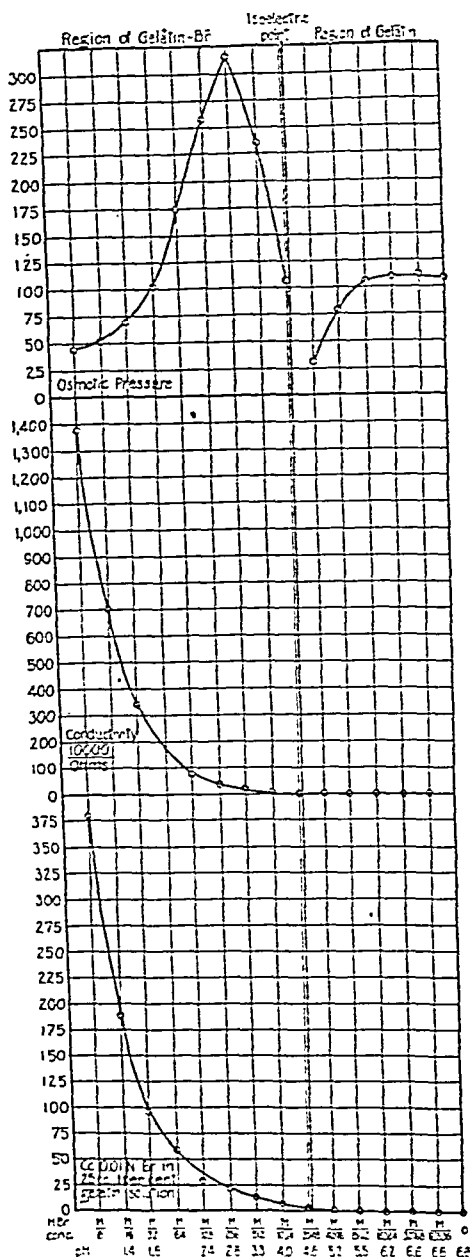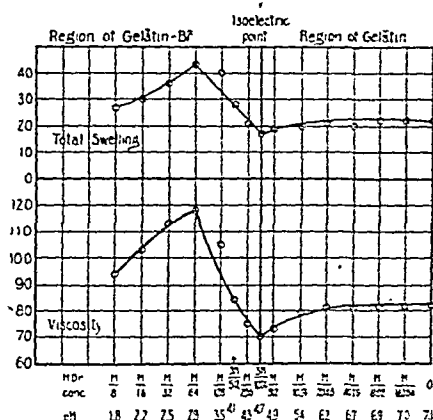HBr of a different concentration.   Then the gelatin is poured on a

FIG. 2. Curves for bromine number (cc. 0.01 N Br in 25 cc. of 1 per cent gelatin solution), conductivity, and osmotic pressure of gelatin in presence of various concentrations of HBr. Abscissæ as in Fig. 1. No parallelism between curve for osmotic pressure and curves for bromine number and conductivity. Curve for osmotic pressure has minimum at pH about 4.6, maximum at pH = 3.3, and then drops. No conclusion can be drawn from such experiments as to the "colloidal" or true character of osmotic pressure. Osmotic pressure measured in height of column of 1 per cent gelatin solution.

filter and the acid allowed to drain off.   The swelling is measured·as
described.· From now on .the method of procedure is different from
that in the previously mentioned experiment.   Instead of melting
the gelatin in 100 cc. of the acid .solution with which it had been
treated, we melt it in 100 cc. of distilled water.   The rest of the deter-
minations—viscosity, osmotic pressure, conductivity, and titration
for Br—are all made with such gelatin.   Moreover, the osmotic pres-

FIGS. 3 and 4. Curves for viscosity and swelling (Fig. 3); bromine number,
conductivity, and osmotic pressure (Fig. 4) of 1 per cent gelatin solution treated
previously with different concentrations of HBr (abscissæ) the acid having been
allowed to drain off.   A 1 per cent solution of the gelatin *in distilled water* is then
prepared, and the osmotic pressure of this gelatin is measured against distilled
water, and conductivity and Br number are determined after 20 hours dialysis
against distilled water.   The curves for osmotic pressure, swelling, viscosity, and
conductivity are parallel to the curve for bromine number from pH = 4.7 to
pH = 2.9 or 3.3 respectively.   The gelatin is free from bromine for pH $\gtrless$ 4.7.

sure was measured against H_2O, thereby allowing more of the free acid
not combined with the gelatin which had not drained off to diffuse
out during the process.   The result of this experiment is represented
in Figs. 3 and 4.   Fig. 4 contains the measurements for osmotic pres-
sure and Br number, and the curves are almost parallel (with the excep-
tion of the value for the osmotic pressure for gelatin treated with м/8
acid).   This parallelism is the missing link which allows us to decide
in favor of the purely chemical and against the colloidal conception
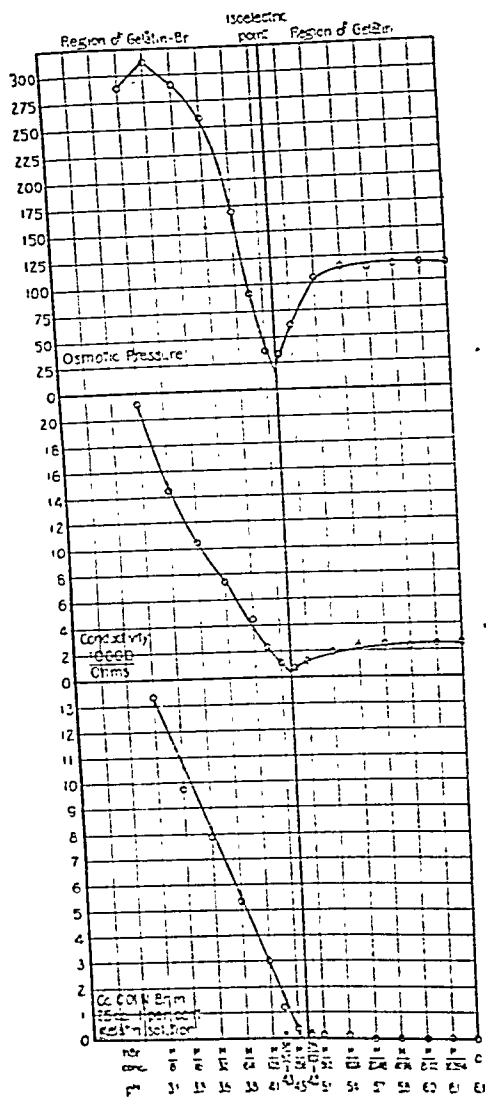
FIG. 4. See explanation under Fig. 3.

of the influence of electrolytes upon the osmotic pressure.   Since the bromine number of gelatin increases parallel with the osmotic pressure (and also the viscosity and the swelling) of gelatin, these properties must depend upon the same variable; namely, the number of gelatin bromide molecules formed.

We are giving the values for the bromine number and for conductivities as actually found by analysis and measurements of resistance.   These values demand, however, a correction owing to the fact that in all cases a certain amount of free HBr was present. The actually measured quantity for the bromine number is in each case the sum of the Br contained in the form of gelatin bromide and of the Br contained in the form of free HBr.   The latter can be calculated from the pH.   This value for the quantity of free HBr should be deducted from the Br numbers given in the curves.   Since the correction thus required would be slight within the limits of pH from 4.7 to 3.6, we have omitted it in this paper.   We may state, however, that the parallelism between the corrected curves for the Br number and those for osmotic pressure is even more perfect than that between our uncorrected curves for bromine number and the curves for osmotic pressure.   The correction necessary for the conductivity curves can be found by measuring the resistance of a HBr solution for each pH in the same measuring cell (with fixed electrodes) which served for the measurements of the resistance of the gelatin bromine solutions.   These corrections are greater than those required for the bromine number, especially for pH < 3.9.   In order to obtain reliable values for conductivity we must use purified gelatin.   Experiments of this kind will be reported in a subsequent paper.   The conductivity measurements will not be considered in this paper.

All three curves for osmotic pressure, viscosity, and bromine number show a drop again after having reached a maximum.   This drop exists in a still more pronounced way in the curves for viscosity and swelling than in that for osmotic pressure, because the gelatin contained more HBr before than after dialysis.   This drop is of great theoretical significance because it shows free HBr is present in excess of the binding capacity of gelatin for HBr.   The free HBr represses the ionization of gelatin bromide on account of the common Br ion and

his causes the drop in the curves for the osmotic pressure of the
gelatin, since the free HBr, being able to diffuse through the collodion
membrane, cannot cause any increase in osmotic pressure. The drop
begins usually when pH becomes <3.3 and the drop is the more con-
siderable the more pH falls below this level.

The correctness of this view is proved by the fact that if we wash
away the traces of free acid left in the capillary spaces between the
particles of gelatin after the process of draining, by perfusing the
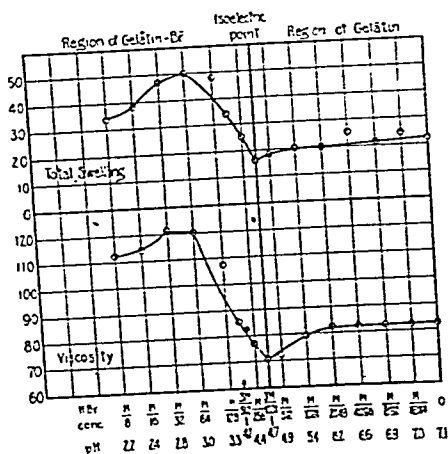
FIGS. 5 and 6. Same curves as in Figs. 3 and 4 except that the gelatin after
the acid had been allowed to drain off was washed once with 25 cc. of H₂O.
Parallelism between curves for Br number, conductivity, osmotic pressure (Fig.
6), viscosity, and swelling from pH = 4.7 to pH = 3.0. No Br found for
pH ≧ 4.7.

gelatin on the filter with 25 cc. of H₂O, and if we allow the water to
drain off also before we make up the gelatin into a 1 per cent solution
in distilled water, the drop will disappear, as is obvious from Figs. 5
and 6. In Fig. 6 the drop has disappeared, the pH going only to
3.3, and in these curves there is as complete a parallelism between
the bromine number and the osmotic pressure of the gelatin solution
as the strict validity of the theory of van't Hoff demands. We still
observe the drop for the curves for viscosity and swelling, but the pH
in these cases falls below 3.3; namely, to 2.2 (Fig. 5).

If instead of giving one washing we give four washings with 25 cc. of H_2O after the acid has drained off, we avoid the low values of pH completely and the drop in the curves for swelling disappears (Fig.
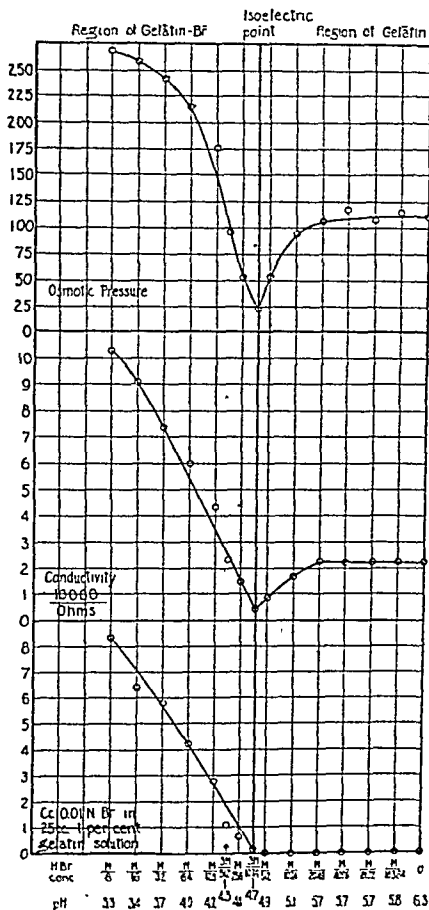

Fig. 6. See explanation under Fig. 5.

7). The parallelism between the curves for bromine number, for osmotic pressure and swelling is now practically complete.

The curves show distinctly that the independent variable is the bromine number. Thus in Fig. 7 this number was slightly in ex-

cess in the gelatin treated with $M/128$ and $M/64$ HBr. Corre-
sponding abnormal values are found in the curves for conductivity,
osmotic pressure, and swelling. The same is shown. in Fig. 8. It
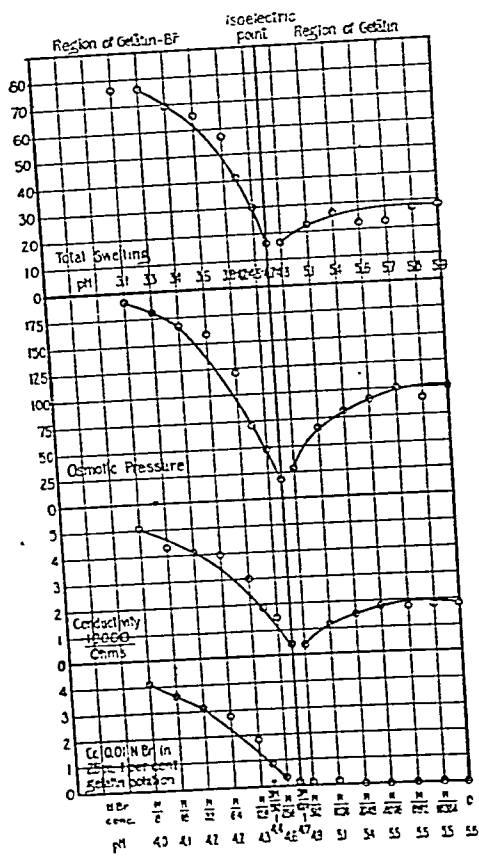
FIG. 7. Same curves as in preceding figures except that gelatin, after the
acid treatment, had been washed four times with H_2O. Explanation as in pre-
ceding figures. Notice that curves are parallel and the gelatin is free from Br
for pH $\leqq$ 4.7.

is, therefore, the Br number which determines the curves, *i.e.* the
amount of gelatin bromide formed. Fig. 8 gives the curves for eight
washings. Again the parallelism between the curves for the Br num-
ber and the other physical properties is obvious.

FIG. 8. Same curves as in preceding figures except that gelatin had been washed eight times with H₂O. Curves parallel and gelatin free from Br for pH ≤ 4.7.

These experiments furnish the proof that the effect of acid (HBr) upon the physical properties of gelatin is the unequivocal function of the amount of gelatin bromide formed; the probable reason for this being that pure gelatin (as it exists at the isoelectric point) is practically insoluble (and undissociated), while gelatin bromide is soluble.

We must now furnish the proof that not only is there a parallelism between the curve for the bromine number on the one hand and the curves for the physical properties of gelatin treated with HBr, but
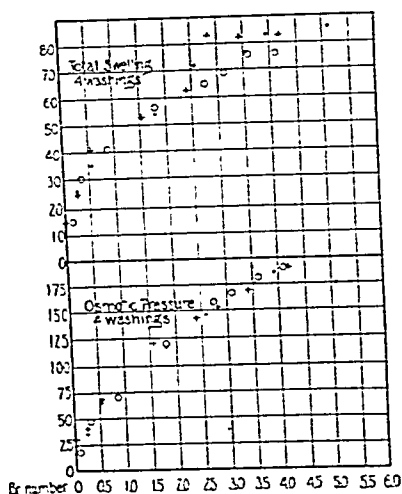

FIG. 9. The abscissæ represent the bromine number, the ordinates the values for osmotic pressure found in three different experiments with 1 per cent gelatin solution previously treated with HBr and washed four times with water. Notice agreement between values.

that to each definite Br number belongs a definite and absolute value for osmotic pressure, conductivity, swelling, and viscosity. We can do this by plotting the results of different experiments with the values for Br numbers as abscissæ and the values for osmotic pressure, swelling, etc., as ordinates. In this case the values obtained for osmotic pressure in the different experiments should differ only within the limits of the accuracy of our measurements.

In Fig. 9 the curves for three different experiments with four washings each are plotted for osmotic pressure and for swelling. The

variations lie within the limit of error. In the experiments plotted in Fig. 10 the number of washings of gelatin varied. In spite of the difference in the treatment we notice that for the same Br number practically the same value of osmotic pressure was found in all experiments. Since the curves for the other properties are parallel to the curve for osmotic pressure, it is not necessary to reproduce all the curves.

We therefore reach the conclusion that the variation of the physical properties of gelatin under the influence of HBr is an unequivocal function of the number of gelatin bromide molecules formed and that colloidal speculations not based on the laws of classical chemistry are neither needed nor warranted.
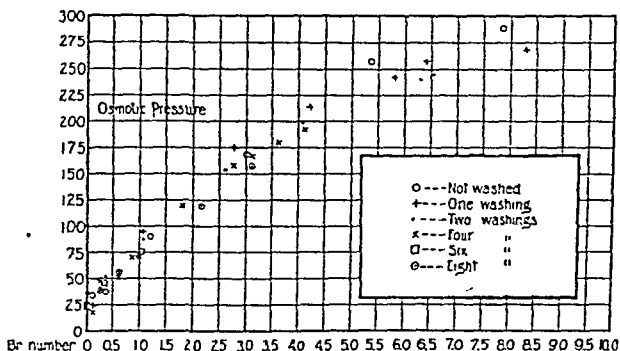

FIG. 10. Abscissæ represent bromine number, ordinates, values for osmotic pressure found in different experiments with 1 per cent gelatin solution previously treated with HBr and washed a different number of times.

IV. *Titration of Gelatin with NaOH.*

The following facts constitute an important link in the proof for a chemical theory of the action of electrolytes (in our case HBr) upon the physical properties of gelatin.

We notice that in all the curves the gelatin was found to be absolutely free from Br at the isoelectric point as well as on the alkaline side from the isoelectric point; *i.e.*, it was free from Br whenever pH $\lessgtr$ 4.7. Yet this gelatin had been treated with HBr. The fact that HBr cannot combine or remain in combination with gelatin for

pH $\lessgtr$ 4.7 is a further proof of the correctness of the purely chemical theory according to which for pH $>$ 4.7 ionized gelatin can only exist as an anion, not capable of holding HBr in combination.

When we titrate 25 cc. of 1 per cent gelatin solution at the isoelectric point with 0.01 N NaOH, we find that it acts as an acid, requiring between 5.25 and 5.5 cc. 0.01 N NaOH for neutralization against phenolphthalein. (The number of cc. 0.01 N NaOH required to neutralize 25 cc. of 1 per cent gelatin we will call the NaOH number of gelatin.) When the pH of common gelatin, not treated with acid, is greater than 4.7 the NaOH number becomes less than 5.25 cc., probably on account of the fact that part of the gelatin exists as a metal gelatinate (probably chiefly Ca gelatinate) owing to ionogenic impurities remaining from the process of manufacture. Our previous papers have shown that at the isoelectric point gelatin is compelled to give off these ionogenic impurities.

On the more acid side from the isoelectric point the gelatin contains Br and the Br number increases with decreasing pH. With the exception of a small fraction this Br is held in combination with the gelatin as can be shown on the basis of titration with NaOH of the gelatin treated previously with HBr and possessing a pH $<$ 4.7. In such a titration the gelatin solution whose pH $<$ 4.7 is gradually rendered more alkaline through the addition of NaOH until finally its pH becomes equal to 4.7, and when that happens all the HBr held in combination with gelatin must be set free. As a consequence in a titration of gelatin bromide with NaOH two acids must be saturated with NaOH, the pure gelatin, and the HBr set free when during the process of titration the gelatin reaches its isoelectric point. It follows from this that the NaOH number found in this case must equal the sum of the Br number of the gelatin plus the NaOH number for gelatin at the isoelectric point; regardless of how the gelatin had been treated before and regardless of the pH for which this rule is tried out.

If we denote a given pH with n, the NaOH number at this pH = n as "$(NaOH)_n$," the NaOH number at the isoelectric point with "NaOH (isoelectric)," and the Br number at pH = n with "Br_n", then the following equation will hold: $(NaOH)_n = NaOH \ (isoelectric) + Br_n$. In Table I, I have selected at random four experiments in which the

NaOH number and the Br number for different values of pH are given. It is easy to show that the equation holds within the limits of accuracy of our experiments. Thus the NaOH number for the iso-electric point is practically a constant in all experiments, namely between 5.25 and 5.5; and this value represents the binding capacity of "pure" gelatin for NaOH; or in other words, the binding capacity of 0.25 gm. of gelatin freed from ionogenic impurities is between 5.25 and 5.5 cc. 0.01 N NaOH, with phenolphthalein as indicator. Thus

TABLE I.

Experiment I. No washings, but made up in H_2O and dialyzed against H_2O.

pH	3.1	3.3	3.6	3.8	4.1	4.3	4.5	4.8	5.1	5.4	5:7	5.8	6.0	6.1
Br number.	13.35	9.75	7.85	5.35	3.0	1.2	0.35	0.1	0.1	0.1	0	0	0	0
NaOH "	18.00	15.00	13.50	11.50	9.0	8.0	7.00	5.0	4.0	2.5	1.75	1.5	1.5	1.5

Experiment II. One washing.

pH	3.3	3.4	3.7	4.0	4.2	4.3	4.4	4.7	4.9	5.1	5.7	5.7	5.8	6.3
Br number.	8.3	6.4	5.8	4.2	2.75	1.05	0.6	0.1	0	0	0	0	0	0
NaOH "	12.5	12.0	11.0	10.0	9.50	7.5	7.0	5.5	4.5	3.0	2.0	1.5	1.5	1.5

Experiment III. Four washings.

pH	3.8	4.0	4.1	4.2	4.3	4.5	4.8	5.0	5.3	5.6	5.7	5.8	5.9
Br number	4.15	3.45	2.85	2.4	0.55	0.25	0	0	0	0	0	0	0
NaOH "	9.00	8.50	8.00	8.0	7.0	6.00	5.5	4.0	2.5	2.25	1.5	1.5	1.5

Experiment IV. Eight washings.

pH	4.1	4.1	4.2	4.2	4.4	4.5	4.5	4.7	4.9	5.2	5.4	5.5	5.6
Br number	3.7	3.1	2.15	0.95	0.6	0.25	0.1	0	0	0	0	0	0
NaOH "	8.0	8.0	7.0	6.5	6.5	6.00	5.5	5.25	4.5	3.25	2.5	2.5	2.0

in Experiment III, for pH = 4.1, $(NaOH)_n$ = 8.0, NaOH (iso-electric) = 5.5, and Br_n = 2.85. 5.5 + 2.85 = 8.35, while $(NaOH)_n$ actually found is 8.0. Table II gives a comparison of the agreement in all the experiments.

In Table II we call the sum of NaOH (isoelectric) + Br_n the calculated and $(NaOH)_n$ the observed value for a given pH. The table shows that the calculated and observed values agree within the limits of the degree of accuracy of the experiments.

We have now to make sure that the HBr measured in these titrations

JACQUES LOEB

is practically the HBr which was in combination with the gelatin, and not free HBr left in the capillary spaces between the particles of powdered gelatin after the treatment of the latter with HBr. The reader will remember that in Experiment I (Table I) the acid was allowed to drain off and that the gelatin solution was afterwards dialyzed for about 20 hours against distilled water. In the other experiments, in addition to this the last traces of acid were removed by one or more additional washings with distilled water. It is a striking fact that for pH ≶ 4.7 the gelatin was always free from Br although it had been treated with HBr. This coincidence of the point where Br begins to appear in the gelatin with the value pH > 4.7 where it theo-

TABLE II.

Experiment I.			Experiment II. (two washings).			Experiment III. (four washings).			Experiment IV. (eight washings).		
	(NaOH)n			(NaOH)n			(NaOH)n			(NaOH)n	
pH	Calcu-lated	Found	pH	Calcu-lated	Found	pH	Calcu-lated	Found	pH	Calcu-lated	Found
4.7	5.5		4.7	5.5		4.7	5.5		4.7	5.5	
4.5	5.85	7.0	4.4	6.1	7.0	4.5	5.75	6.0	4.5	5.35	5.5
4.3	6.7	8.0	4.2	8.25	9.5	4.2	7.9	8.0	4.5	5.5	6.0
4.1	8.5	9.0	4.0	9.7	10.0	4.1	8.3	8.0	4.4	5.85	6.5
3.8	10.8	11.5	3.7	11.3	11.0	4.0	9.0	8.5	4.2	6.2	6.5
3.6	13.3	13.5	3.4	11.9	12.0	3.8	9.6	9.0	4.2	7.4	7.0
3.3	15.2	15.0	3.3	13.8	12.5				4.1	8.3	8.0
3.1	18.8	18.0							4.1	8.9	8.0

retically should begin to appear speaks already against the assumption that the Br number is the expression of free HBr not drained or dialyzed or washed off.

The direct proof lies, however, in a comparison between the pH and the Br number. We select at random in Experiment III (Table I) and the Br number found in 25 cc. of gelatin solution is for pH = 4.0. The Br number found in 25 cc. of gelatin solution is for this pH 3.45 cc. 0.01 N Br. In the form of free acid this Br number would represent a hydrogen ion concentration of $\frac{3.45}{2,500}$ N, which is approximately $\frac{1}{700}$ N, while the actual normality of the gelatin solution was $\frac{N}{10,000}$; i.e., less than one-tenth of $\frac{N}{700}$ (about 7 per cent). Hence more than 90 per cent of the HBr existed in chemical com-

bination with the gelatin and the small amount of free acid found was probably due to hydrolytic dissociation of gelatin bromide or to a trace of HBr not removed. This quantity of free acid is the correction of the value for the bromine number referred to in an earlier part of this paper. When, however, the free HBr is not washed off, as was the case in the experiment represented in Fig. 1, or when the free acid is only incompletely removed, the value $(NaOH)_n$ will represent, of course, more or less free acid and in this case the discrepancy between $(NaOH)_n$ and the pH found will be correspondingly smaller (see Fig. 1). This, however, manifests itself by the fact that a further rise in the Br number is no longer accompanied by a corresponding rise or is accompanied by a drop in the curves for osmotic pressure, swelling, and viscosity.

We may, therefore, consider it as proved that the bromine numbers given in this paper represent practically the HBr held in chemical combination by the gelatin with the exception of the small amount to be deducted owing to the presence of free HBr which can be calculated from the pH.

V. Theoretical Remarks.

Our experiments show that the influence of hydrobromic acid upon the physical properties of gelatin has a purely chemical or stoichiometrical basis. Gelatin and probably all proteins and amphoteric colloids behave as if they were merely amphoteric electrolytes capable of adding a H or OH ion.

Whether a protein adds an acid or a base depends on the hydrogen ion concentration; when the hydrogen ion concentration exceeds a critical point (which for gelatin is $C_H = 2.10^{-5}$), the gelatin will add acid; when it is lower it will add base. This critical hydrogen ion concentration is the isoelectric point. When gelatin is at the isoelectric point, it is free from ionogenic impurities and this "pure" gelatin is practically insoluble and hence can have no osmotic pressure, and, moreover, all the properties which depend upon its solubility are a minimum. When it is transformed into a salt by the addition of an acid (or a base) it becomes soluble, provided it is in combination with a monovalent ion, like Br or Na, etc. When pure gelatin is

JACQUES LOEB

transformed into a salt with monovalent anion or cation, all those properties which depend upon the number of gelatin molecules in solution increase with the proportion of gelatin salt formed, the maximum being reached when all the insoluble gelatin is transformed into soluble gelatin salt. This explains the parallelism between the curves for the bromine number of gelatin treated with HBr and the curves for the osmotic pressure of the solution. These molecular data must explain also the parallelism between the curves for viscosity and swelling with that of the bromine number.

The view taken in this paper that the osmotic pressure of protein solutions obeys the laws of classical physical chemistry is shared by one of the greatest authorities in this field, namely Sörensen.[9] Sörensen worked on egg albumin of a well defined composition, which necessitated the investigation of the osmotic pressure of gelatin in the presence of ammonium sulfate. In spite of the great theoretical and experimental difficulties, which only a master like Sörensen could succeed in overcoming, he arrived at constant values for the osmotic pressure and the molecular weight of egg albumin.

He states:

"The properties of colloidal solutions can be most efficiently inquired into by application, as far as possible, of the same views and methods as those generally applied to true solutions. . . . [10]

"Colloidal chemistry in the shape which has been given it by its energetic champion Wo. Ostwald offers, no doubt, to protein study a system organized with great talent, but exact experimental investigation has not been able to keep up with the systematic treatment, and therefore the value of the contents does not always correspond with the perfection of the system itself. Thus I disagree with Ostwald, who . . . warns us against a comparison of the circumstances in colloidal and real solutions. In the case of albumin solutions, and doubtless also several other typical emulsoid systems, such a comparison between the properties of the colloidal solution and those of a real solution is of the greatest significance for the right understanding of the character of the colloidal solution. Indeed, I think it is even possible to go one step further and to say that the study of real solutions may derive considerable advantage from the results which an exact research of well-defined protein solutions can give, the colloidal character of these permitting the use of research-methods—I refer especially to the use of

9 Sörensen, S. P. L., Compt. rend. trav. Lab. Carlsberg. 1917, xii.
10 Sörensen, Compt. rend. trav. Lab. Carlsberg. 1917, xii, 369.

semi-permeable membranes—, which, when dealing with real solutions, can only
be used in exceptional cases and under especial circumstances."[11]

SUMMARY.

1. The method of removing the excess of hydrobromic acid after
it has had a chance to react chemically with gelatin has permitted
us to measure the amount of Br in combination with the gelatin.
It is shown that the curves representing the amount of bromine bound
by the gelatin are approximately parallel with the curves for the
osmotic pressure, the viscosity, and swelling of the gelatin solution.
This proves that the curves for osmotic pressure are an unequivocal
function of the number of gelatin bromide molecules formed under
the influence of the acid.   The cc. of 0.01 N Br in combination with
0.25 gm. of gelatin we call the bromine number.

2. The explanation of this influence of the acid on the physical prop-
erties of gelatin is based on the fact that gelatin is an amphoteric
electrolyte, which at its isoelectric point is but sparingly soluble in
water, while its transformation into a salt with a univalent anion
like gelatin Br makes it soluble.   The curve for the bromine number
thus becomes at the same time the numerical expression for the num-
ber of gelatin molecules rendered soluble, and hence the curve for
osmotic pressure must of necessity be parallel to the curve for the
bromine number.

3. Volumetric analysis shows that gelatin treated previously with
HBr is free from Br at the isoelectric point as well as on the more
alkaline side from the isoelectric point (pH $\gtrless$ 4.7) of gelatin.   This
is in harmony with the fact that gelatin (like any other amphoteric
electrolyte) can dissociate on the alkaline side of its isoelectric point
only as an anion.   On the more acid side from the isoelectric point
gelatin is found to be in combination with Br and the Br number
rises with the pH.

4. When we titrate gelatin, treated previously with HBr but pos-
sessing a pH = 4.7, with NaOH we find that 25 cc. of a 1 per
cent solution of isoelectric gelatin require about 5.25 to 5.5 cc. of 0.01
N NaOH for neutralization (with phenolphthalein as an indicator).

[11] Sörensen, *Compt. rend. trav. Lab. Carlsberg*, 1917, xii, 5–6.

This value which was found invariably is therefore a constant which we designate as "NaOH (isoelectric)." When we titrate 0.25 gm. of gelatin previously treated with HBr but possessing a pH<4.7 more than 5.5 cc. of 0.01 N NaOH are required for neutralization. We will designate this value of NaOH as "$(NaOH)_n$," where n represents the value of pH. If we designate the bromine number for the same pH as "Br_n" then we can show that the following equation is generally true: $(NaOH)_n = NaOH$ (isoelectric) $+ Br_n$. In other words, titration with NaOH of gelatin (previously treated with HBr) and being on the acid side of its isoelectric point results in the neutralization of the pure gelatin (NaOH isoelectric) with NaOH and besides in the neutralization of the HBr in combination with the gelatin. This HBr is set free as soon as through the addition of the NaOH the pH of the gelatin solution becomes equal to 4.7.

5. A comparison between the pH values and the bromine numbers found shows that over 90 per cent of the bromine or HBr found was in our experiments in combination with the gelatin.

MONOGRAPHS OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

Under the head of *Monographs of The Rockefeller Institute for Medical Research* are published from time to time scientific papers which are so extensive, or require such elaborate illustrations, as to render them unsuitable for current periodical issues. *Monographs* will be sent post paid on application, at $1.00 each, payable in advance by check or money order.

Monograph No. 1. (Issued June 30, 1910.)

SIMON FLEXNER and J. W. JOBLING. Studies upon a transplantable rat tumor. Plates 1–16. J. W. JOBLING. The biology of a mixed tumor of the rat. MAUD L. MENTEN. Experiments on the influence of radium bromide on a carcinomatous tumor of the rat. J. W. JOBLING. Spontaneous tumors of the mouse. Plates 17–28. J. W. JOBLING. Transplantation experiments in *Macacus rhesus* with a carcinomatous teratoma from man.

Monograph No. 2. (Issued February 15, 1911.)

WILLIAM TRAVIS HOWARD and OSCAR T. SCHULTZ. Studies in the biology of tumor cells. Plates 1–6.

Monograph No. 3. (Issued March 15, 1911.)

BENJAMIN T. TERRY. Chemo-therapeutic trypanosome studies with special reference to the immunity following cure.

Monograph No. 4. (Issued June 24, 1912.)

FRANCIS W. PEABODY, GEORGE DRAPER, and A. R. DOCHEZ. A clinical study of acute poliomyelitis. Plates 1–13.

Monograph No. 5. (Issued September 27, 1915.)

J. P. SIMONDS. Studies in *Bacillus welchii*, with special reference to classification and to its relation to diarrhea.

Monograph No. 6. (Issued January 31, 1916.)

JAMES L. STODDARD and ELLIOTT C. CUTLER. Torula infection in man. Plates 1–9.

Monograph No. 7. (Issued October 16, 1917.)

OSWALD T. AVERY, H. T. CHICKERING, RUFUS COLE, and A. R. DOCHEZ. Acute lobar pneumonia. Prevention and serum treatment. Plates 1–3.

Monograph No. 8. (Issued July 31, 1918.)

ERNEST C. DICKSON. Botulism. A clinical and experimental study. Plates 1–10.

Monograph No. 9. (Issued January 21, 1919.)

JAMES HOWARD BROWN. The use of blood agar for the study of streptococci. Plates 1–34.

Monograph No. 10. (In press.)

WILLIAM G. MacCALLUM. The pathology of the pneumonias in the United States Army camps during the winter of 1917–18. Plates 1–53.

Monograph No. 11. (In press.)

FREDERICK M. ALLEN, EDGAR STILLMAN, and REGINALD FITZ. Total dietary regulation in the treatment of diabetes.

·THE JOURNAL·

OF

·GENERAL PHYSIOLOGY

Owing to the difficulties placed in the way of the printing trades by war conditions, *The Journal of General Physiology* desires to cooperate with the printer by eliminating unnecessary labor in the setting up of manuscripts. To accomplish this object, parts of the papers to be printed in different styles of type should be separated in the manuscript. For example, every experiment, table, or quotation of over five lines should begin on a new sheet. When the text is resumed, a fresh sheet should be started. Separate sheets should be used·for the running head-lines, title, author, foot-notes, and bibliography. The name of the laboratory where the work was done together with the words, "(Received for publication,   , 1918)," should also be written on a separate sheet.

The entire manuscript should be copied with triple spacing.

In general the forms for headings, tables, references, etc., outlined in the "Suggestions for the preparation of manuscripts," issued by the Publication Department, should be followed. A copy of the "Suggestions" will be found on the back pages of the 25-Volume Index of *The Journal of Biological Chemistry*. We are glad to mail additional copies to contributors.

The *Journal* requests that, when possible, authors prepare their manuscripts in conformity with these suggestions.

INFLUENCE OF ELECTROLYTES UPON THE VISCOSITY OF DOUGH.

BY L. J. HENDERSON, W. O. FENN,* AND EDWIN J. COHN.*

(*From the Wolcott Gibbs Memorial Laboratory, Harvard University, in Cooperation with the Division of Food and Nutrition, Medical Department, U. S. Army.*)

The rising of bread and the quality of the baked loaf depend upon the nature of the dough and especially upon such of its properties as

CORRECTION.

induced by these new methods.

practice are explained by these results.

Measurement of the Viscosity of Dough.

The property that we shall call viscosity is quantitatively measured in the experiments reported by the resistance of dough to stirring. 12 gm. of dough are packed in the torsion viscosimeter represented, full size, in Fig. 1.

The viscosimeter is placed in a thermostat at 30°C. and the dough allowed to stand 10 minutes in order to bring it to the temperature of

* Lieutenant. Sanitary Corps, U. S. Army.

the bath. The paddles are forced through the dough by the fall of 150 gm. through 2 meters. The rate of the falling of the weight is recorded and measures the viscosity of the dough.

BUCKET OF WEIGHTS
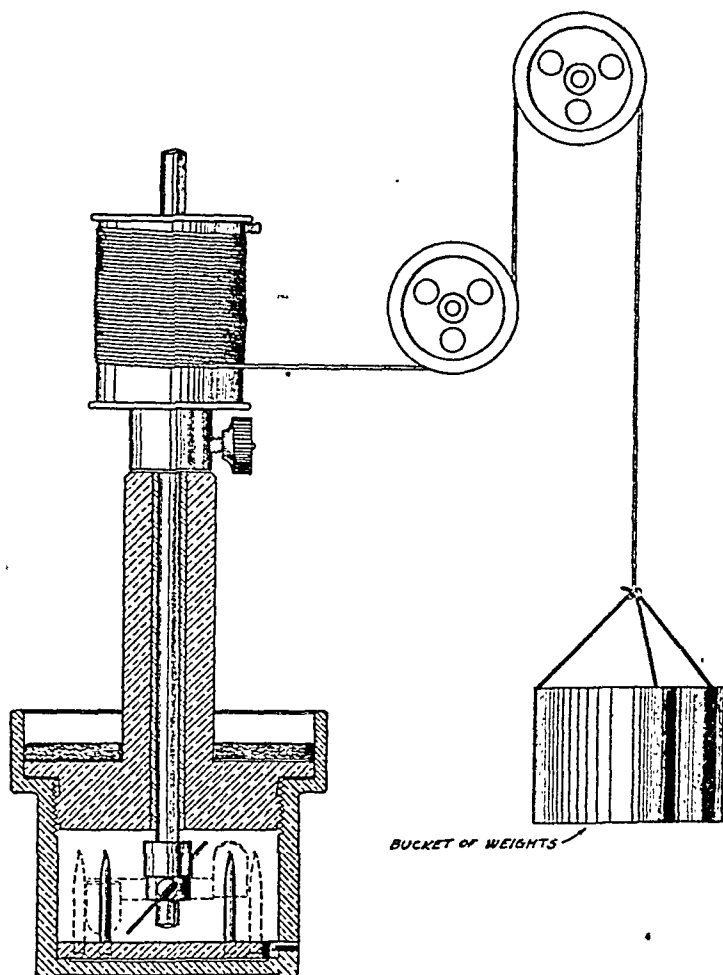
FIG. 1.

The turning of the dough inside the viscosimeter is prevented by four equidistant pins. It has been found convenient to attach the pins to a plate rather than to the bottom of the viscosimeter to facili-

TABLE I.

Viscosity of Dough at Different Hydrogen Ion Concentrations. Time in Minutes of Fall of Weight in Torsion Viscosimeter.

Estimated pH of dough.	Mols of HCl per 1,000 gm. of flour.	Experiment 725. 1,000 gm. of Flour A, 600 cc. H₂O.	Experiment 664. 1,000 gm. of Flour B, 590 cc. H₂O.	Experiment 728. 1,000 gm. of Flour D, 580 cc. H₂O.	Experiment 747. 1,000 gm. of Flour M, 540 cc. H₂O.
		min.	*min.*	*min.*	*min.*
3.65	0.054	.			15.8
3.80	0.048	16.2 16.4			
3.85	0.046			15.7 14.0	
3.90	0.043				12.8 11.8
4.05	0.040		14.0 12.5		
4.20	0.036	15.0 12.9 14.5			
4.20	0.035			8.3 11.3 10.1	
4.30	0.032				11.1 10.7
4.40	0.030		10.6 12.3		
4.65	0.024	13.9 14.2 12.1			
4.70	0.023			8.4 10.1 9.6	
4.75	0.022				10.1 9.6
4.85	0.020		10.0 9.2		
5.20	0.012	14.2 17.2			

TABLE I—*Concluded*.

Estimated pH of dough.	Mols of HCl per 1,000 gm. of flour.	Experiment 725. 1,000 gm. of Flour A, 600 cc. H₂O.	Experiment 664. 1,000 gm. of Flour B, 590 cc. H₂O.	Experiment 728. 1,000 gm. of Flour D, 580 cc. H₂O.	Experiment 747. 1,000 gm. of Flour M, 540 cc. H₂O.
		min.	min.	min.	min.
5.20	0.012			10.0 11.0	
5.22	0.011				11.9 11.6
5.25	0.010		13.7 11.0		
5.50	0.005		12.7 14.3		
5.80	0	20.0 20.2	18.1 14.3	15.5	15.2
	NaOH				
6.50	0.01		19.4 24 1		
7.27	0.02		31.0 25.0		
7.62	0.03		40.2		

tate the cleaning of the apparatus. A small pin keeps the plate from turning.

The flour is best gathered together with a stirring rod in the beaker into which the various solutions are run. The volume of water, including that containing dissolved electrolytes, added to the flour is recorded in cc. of H₂O per 1,000 gm. of flour. The quantity of electrolytes is recorded in mols per 1,000 gm. of flour. The small size of the doughs used in these experiments made it necessary further to work them by hand. This was always done by the same experimenter and for precisely 2 minutes. The solutions of electrolytes of different strength were added with the water.

No great accuracy is possible in carrying out such measurements, first, because a very exact control of the water content is almost impossible without undue expenditure of time, and secondly, because slight differences in the packing of the viscosimeter are not without serious influence. However, the data will show the degree of accuracy
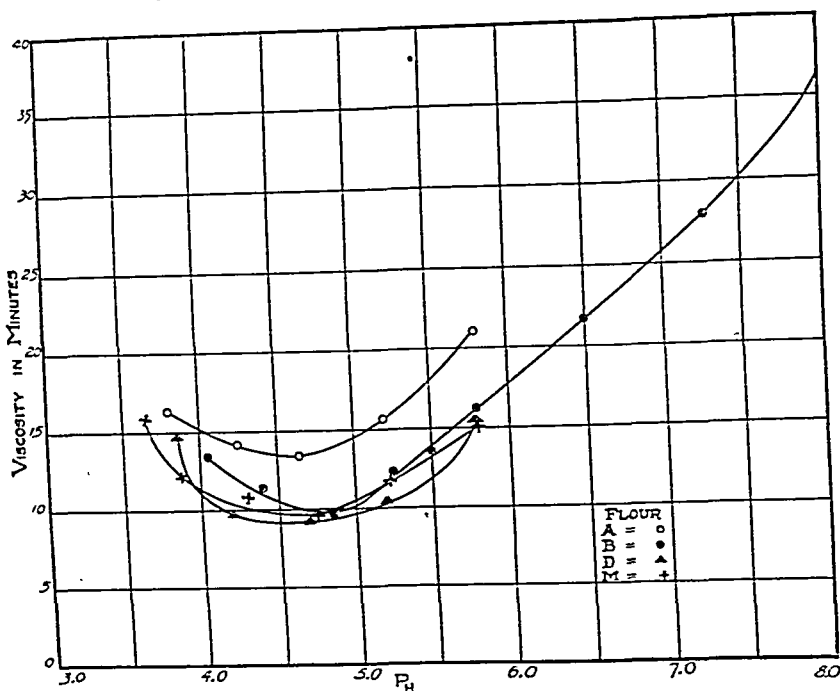
FIG. 2.

of the observations and, as we believe, justify such use of them in drawing conclusions as is at present necessary.

Effect of Hydrogen Ion Concentration upon the Viscosity of Dough.

Our first series of experiments concerns the influence of hydrogen ion concentration upon the viscosity of dough prepared from four different flours. Differences of reaction were produced by the addition of solutions of hydrochloric acid and sodium hydroxide and estimated with the help of the concentration cell. The varied character of the four flours is indicated by the difference in the water content of their approximately isoviscous doughs.

The results clearly show that a single measurement of viscosity possesses no great significance (Table I and Fig. 2). Nevertheless it is evident that with variation in hydrogen ion concentration viscosity passes through a minimum a little on the acid side of pH 5. Jessen-Hansen[1] has found pH 5 to be the best hydrogen ion concentration in bread making and it is also. as we have observed, the most favorable reaction for the rising of dough.

[1] Jessen-Hansen, H., *Compt. rend. trav. Lab. Carlsberg*, 1911, v, no. 10.

Effect of Salts upon the Viscosity of Dough.

The second series of experiments concerns the influence of certain salts upon the viscosity of dough (Tables II and III and Fig. 3)

TABLE II.

Viscosity of Dough in the Presence of Different Concentrations of Salts. 1,000 Gm. of Flour B; 590 Cc. of Water. Time in Minutes; Torsion Viscosimeter.

Mols of salt.	Experiment 671. NaCl	Experiment 684. Na lactate.*	Experiment 680. Na₂SO₄	Experiment 680. MgSO₄
	min.	*min.*	*min.*	*min.*
0	13.7 13.4 15.7	12.4 11.3		
0.059	10.8 11.4	9.2 ·8.9	10.7 11.2	8.6
0.118	11.0 9.7		·	6.9 6.8
0.177	13.9 11.0 10.0	7.6 8.4	·	·
0.194			6.6 6.7	
0.236		·		4.3 3.7
0.295	13.4 13.3		4.5 5.0	
0.354		5.9 ·7.0		3.0 3.5
0.590	13.9 18.9 13.9	7.6 7.6		4.9 4.4

* The moisture of the flour slightly increased the water content and decreased the viscosity of the doughs in Experiment 684. This is shown by the viscosity of dough containing no Na lactate. A mixture of 49 parts of Na lactate and 1 part of lactic acid approximately isohydric with flour was used.

These experiments show that the addition of a small amount of salt to dough tends to diminish viscosity. As the concentration of the salt increases, however, there is commonly, and probably with

TABLE III.

Viscosity of Dough in the Presence of Different Concentrations of Salts. 1,000 Gm. of Flour M; 550 Cc. of Water. Time in Minutes; Torsion Viscosimeter.

Mols of salt.	Experiment 744. KBrO$_3$	Experiment 742. NH$_4$Cl	Experiment 750. MgCl$_2$
0	min. 14.2	min. 12.1 12.7	min.
0.064	11.9 10.5	11.4 12.3	12.6 11.7
0.137	11.0 13.0	10.0 11.4	11.2 10.8
0.275	11.8 10.4	10.4 13.7 13.7	10.7 11.8
0.550		14.4 15.6	18.5 18.7
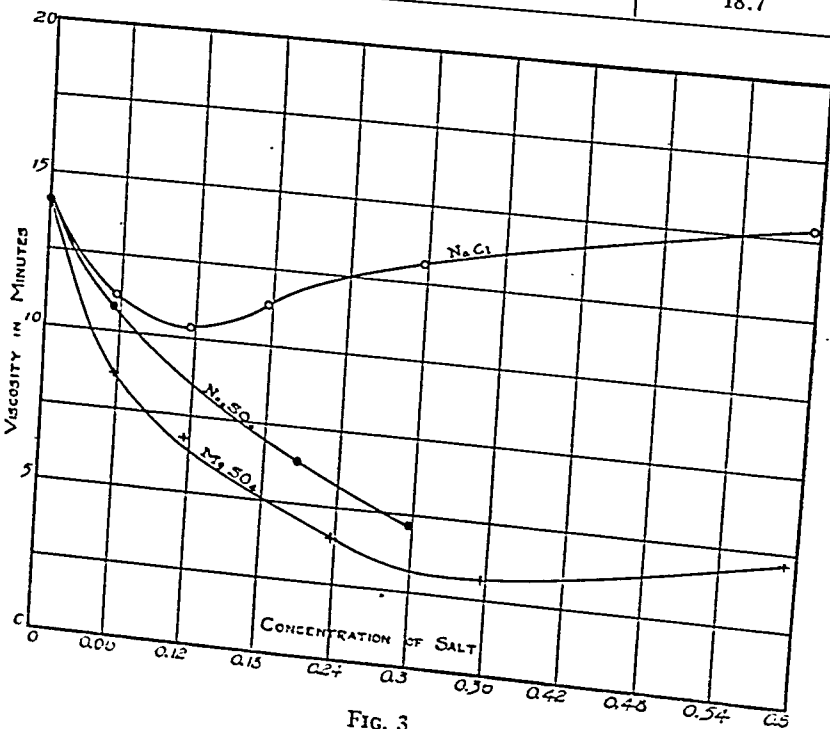
FIG. 3.

sufficiently high concentration always, a later rise in viscosity. Among the salts which we have studied the sulfates of sodium and magnesium have the most marked effect. In these cases it is sufficient to handle the dough in order to note a certain "slackness" or diminished firmness.

Effect of Salts upon the Viscosity of Dough at Different Hydrogen Ion Concentrations.

The third series of experiments concerns the simultaneous influence of acid or alkali and salt upon the viscosity of dough. The effect of different concentrations of a salt at two hydrogen ion concentrations is illustrated in Table IV and Fig. 4; the effect of the same con-

TABLE IV.

Viscosity of Dough in the Presence of Different Concentrations of NaCl at Different Hydrogen Ion Concentrations. (Time in Minutes.)

Experiment No.	Estimated pH of dough.	Mols of HCl per 1,000 gm. of flour.	1,000 gm. of Flour B; 590 cc. H₂O. Mols of NaCl.									
			0	0.049	0.059	0.118	0.147	0.177	0.246	0.295	0.492	0.590
			min.	min.	min.	min.	min.	min.	min.	min.	min.	min.
671	‾5.8	0.0	13.7 13.4 15.7		10.8 11.4	11.0 9.7		13.9 11.0 10.0		13.4 13.3		13.9 18.9 13.9
673	4.8	0.02	13.3	11.7 10.4			8.7		9.1 9.4		8.7 9.5	

centration of a salt at different hydrogen ion concentrations is shown in Table V and Fig. 5.

These experiments show that the influence of salts upon viscosity is variable with the hydrogen ion concentration. Particularly important is the fact that at a more acid range of reaction sodium chloride greatly depresses viscosity while calcium chloride has its effect in a less acid range. The great effect of the sulfates, however, may be recognized over a wide range of reaction.

For the present we shall postpone a discussion of the theoretical bearings of these experiments, which are often obscure, and may best be taken into account in connection with observations upon the properties of gluten soon to be published from this laboratory.

The importance of these results in bread making depends upon their relation to a variety of other facts. First, it has long been known that bread possesses a distinctly acid reaction. This is due to the fact that beginning with an acid dough of hydrogen ion concentration 10^{-6} N or more, the process of fermentation produces an augmentation of acidity. The observations of Jessen-Hansen in the Carlsberg Laboratory confirm this fact by quantitative measurements
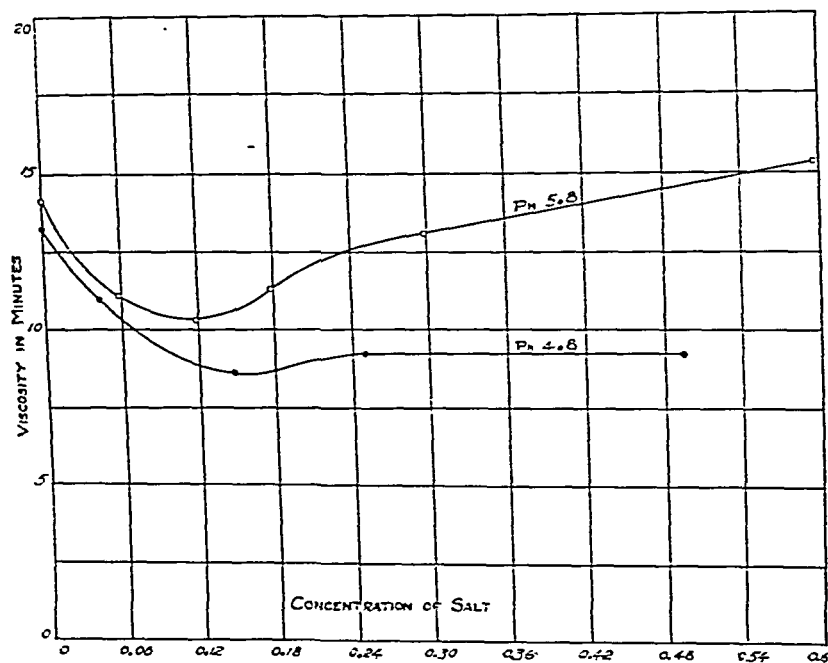
FIG. 4.

and lead their author to the conclusion that about 10^{-5} N hydrogen ion concentration is the most favorable for bread making. Observations of our own (except with a very weak flour) agree with those of Jessen-Hansen. We have also made numerous measurements of the hydrogen ion concentration of bread procured in the market, which are entirely consistent with this view.

Second, our studies reported in another paper of the rising of dough and the carbonic acid production during fermentation

TABLE V.

Viscosity of Dough in the Presence of Salts at Different Hydrogen Ion Concentrations.
Time in Minutes; Torsion Viscosimeter.

Estimated pH of dough.	Mols of HCl per 1,000 gm. of flour.	Experiment 664. 1,000 gm. of Flour B. 590 cc. H₂O.	Experiment 672. 1,000 gm. of Flour B. 590 cc. H₂O. NaCl 0.59 mols.	Experiment 677. 1,000 gm. of Flour B. 590 cc. H₂O. CaCl₂ 0.393 mols.	Experiment 683. 1,000 gm. of Flour B. 590 cc. H₂O. NaCl 0.314 + CaCl₂ 0.079 mols.	Experiment 681. 1,000 gm. of Flour B. 590 cc. H₂O. Na₂SO₄ 0.196 mols.
2.70	0.118					12.4 8.8
3.00	0.088		16.4			
3.55	0.059		12.8 11.0		17.3	5.4 6.0
3.85	0.047		6.3 5.8		10.3 7.6 10.3	5.2 5.2 4.5
4.05	0.040	14.0 12.5				
4.25	0.035		8.3 7.4		8.7 9.2	4.5 5.3 6.2 4.4
4.40	0.030	10.6 12.3				
4.65	0.024		9.8 8.3	16.9	10.2	4.9 4.7
4.85	0.020	10.0 9.2				
5.20	0.012		13.7 12.5	14.9 14.6		
5.25	0.010	13.7 11.0				
5.50	0.006			14.2 13.2	13.3 13.2	
5.55	0.005	12.7 14.3				
5.80	0	18.1 14.3	18.3 14.8	11.8 13.4		6.7 6.6
6.50	NaOH 0.010	19.4 24.1				
6.60	0.012		17.1 16.4	15.5 15.9		
7.27	0.020	31.0 25.0				
7.70	0.024					12.7
8.20	0.030	40.2				

indicate that the best results are obtained near the point of minimum viscosity.

Finally, the favorable results in the baking industry obtained by the use of calcium salts in the absence of acid and of acid in the absence of calcium salts also indicate that it is advantageous to employ dough at a relatively low viscosity, or, speaking more correctly, to
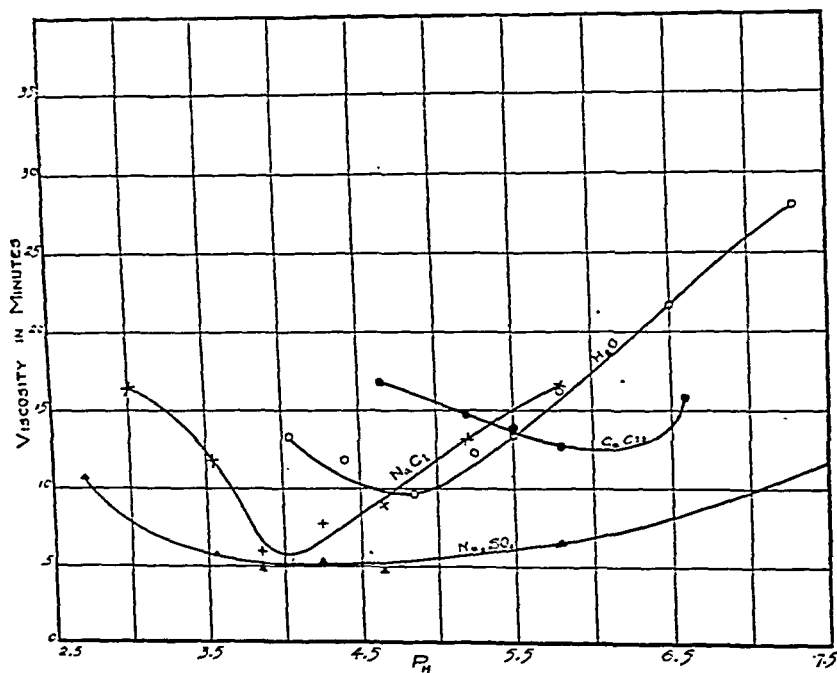
FIG. 5.

reduce whatever quantity of gluten may be present to a condition in which its viscosity is low.

As far as our investigations of this and related questions have proceeded, they indicate that in bread making the action of acids, bases, and salts, perhaps with the exception of potassium bromate, is favorable chiefly through the effect upon viscosity. Any influence upon the activity of yeast appears to be a matter of secondary importance.

COMPARATIVE STUDIES ON RESPIRATION.

VI. Increased Production of Carbon Dioxide Accompanied by Decrease of Acidity.

By MARIAN IRWIN.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

In a previous paper[1] I have shown that high concentrations of ether greatly increase the production of CO_2 by animals and that this is associated with irreversible changes ending in death. It might be thought that this increase could be explained on the ground that the process of death is often accompanied by the production of lactic acid, and this might displace CO_2 from carbonates and bicarbonates present in the tissues. The resulting evolution of CO_2 might be mistaken for increased respiration.

In order to obtain some light on this question it seemed desirable to experiment upon an organism in which death is not accompanied by an increase in acidity. Suitable material for such experiments is furnished by the petals of many flowers containing natural indicators, whose colors show that during the process of death the cell contents become less acid.[2]

Two species of *Salvia* were used in these experiments, *Salvia involucrata* and *Salvia splendens*. The natural indicators contained in the petals were calibrated by placing the petals in boiling water and then transferring them to buffer solutions of known pH value. They remained in the buffer solutions until complete penetration had taken place (this required only a few minutes). As the color of the indicator in various buffer solutions was known, it was a simple matter to determine the changes in acidity[3] which take place in flowers immersed

[1] Irwin, M., *J. Gen. Physiol.*, 1918–19, i, 209.

[2] Haas, A. R., *J. Biol. Chem.*, 1916, xxvii, 233.

[3] The petals contain some cells which lack the indicator; their acidity was consequently not determined.

in a 7.3 per cent (by volume) solution of ether.   These changes are shown in Table I.

The CO_2 output of *Salvia involucrata* was measured by the methods used in the previous investigation.   The petals[4] were immersed in tap water and the normal respiration was determined.   The petals were then immersed in a solution of 7.3 per cent ether (by volume) and new determinations were made.   The results are shown in Fig. 1.

Objection might be made to submerging the petals in liquid on the ground that this condition is abnormal and that gas might be given off more rapidly through the stomata when ether is present (as the result of changes in surface tension).   For this reason it seemed de-

TABLE I.

Changes in the pH Value of Salvia Petals during the Process of Death Caused by Immersion in Solution of 7.3 Per Cent Ether (by Volume).

Length of exposure to ether.	pH value.	
	Salvia involucrata.	*Salvia splendens.*
min.		
0	4.5	3
3	5.0	
5	6.0	
6.5		5
7	7 to 8	
10		7 to 8

sirable to repeat the experiments by the method described by Osterhout.[5]   As flowers of *Salvia involucrata* were no longer available *Salvia splendens* was used.   The corollas were placed in a glass tube, the normal respiration was measured, and sufficient ether was then introduced into the apparatus to saturate the air with ether vapor at 20°C.[6]   The rate of respiration was then determined at frequent intervals.   In respect to the per cent of increase produced by ether the results agreed closely with those shown in Fig. 1.

[4] In all the experiments the entire corolla was used, care being taken to detach it with as little injury as possible.

[5] Osterhout, W. J. V., *J. Gen. Physiol.*, 1918–19, i, 17.

[6] The petals were at no time in contact with liquid.

Experiments were made to determine whether exposure to ether
causes an increase in the consumption of oxygen. For this purpose
the apparatus shown in Fig. 2 was employed. The petals were placed
in a glass tube A, which was connected at one end (by tubes of glass
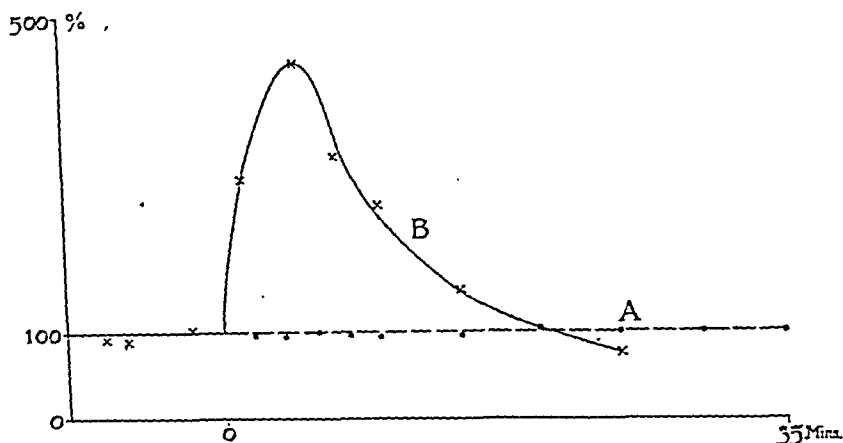
FIG. 1. Curves showing the respiration of *Salvia* petals first in tap water (hori-
zontal line) and then in 7.3 per cent ether. Time is reckoned from the beginning
of exposure to ether. The respiration of a control in tap water is shown by the
broken line. The normal rate (which is taken as 100 per cent) corresponds to a
change in pH value from 8.0 to 7.7 in 193 seconds. Temperature, 20° ± 1°C.
Probable error less than 1.7 per cent of the mean. Curve A, average of six ex-
periments; Curve B, average of four experiments.
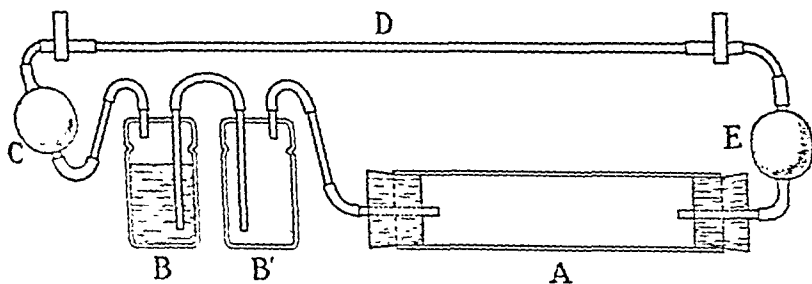
FIG. 2. Apparatus for the determination of oxygen consumption. Petals were
placed in A; by alternate compression of the bulbs C and E the air in the appara-
tus was made to circulate. At the close of each experiment the tube D was
removed and its contents were analyzed for oxygen.

and rubber)[7] to a bottle B, and a safety bottle B'.   B contained strong H_2SO_4 and was connected to the rubber syringe bulb C.   A was connected at the other end to the rubber syringe bulb E and to the long narrow glass tube D.   When the bulbs C and E[8] were compressed, the air passed through A, B', and B into C, and thence through D and E back to A.

Petals were placed in A and by alternately compressing the bulbs C and E a constant circulation of air was maintained for 10 minutes, after which the tube D was clamped off at both ends and the contained gas was analyzed for oxygen.[9]   After making determinations of the normal oxygen consumption in this manner the petals were removed and exposed for 3 minutes to air saturated with ether vapor at $18° \pm 1°C$.   They were then placed in A and a new determination was made.   The H_2SO_4 removed the ether so that it could not interfere with the subsequent analysis.

Since the concentration of ether[10] was less than in the experiments previously described, the oxygen consumption could not be expected to equal the production of CO_2 in these experiments.   Nevertheless the average of four experiments showed that ether increased the consumption of oxygen to 2.3 times the normal.   The CO_2 production during the same time (in a saturated solution of ether), as measured by the methods previously discussed, amounted to three times the normal.

It is therefore evident that in this instance a high concentration of ether causes an increase in the consumption of oxygen as well as in the production of CO_2 and that this is accompanied by a decrease in the acidity of the cells.   It is therefore possible that in other cases where the acidity of the cell cannot be directly measured, the evolution of CO_2 observed under the influence of ether is not wholly due to the displacement of CO_2 from carbonates and bicarbonates stored in the tissues.

[7] All connections were made with as little rubber tubing as possible, and care was taken to make all joints air-tight.

[8] These are provided with valves.

[9] The method employed was that described by Osterhout, W. J. V., *Am. J. Bot.*, 1918, v, 105.

[10] The concentration of ether constantly diminished during the 10 minutes.

SUMMARY.

In petals of *Salvia* high concentrations of ether cause an increase in oxygen consumption and in the production of CO_2, while at the same time a decrease occurs in the acidity of the cell contents.

DECREASE OF PERMEABILITY AND ANTAGONISTIC EFFECTS CAUSED BY BILE SALTS.

By W. J. V. OSTERHOUT.

(*From the Laboratory of Plant Physiology, Harvard University, Cambridge.*)

Agents which increase permeability have long been known but the discovery of substances which have the opposite effect is comparatively recent.[1] The number of such substances known at present (especially organic substances) is very small and it is therefore of interest to find that bile salts possess this property.

The discovery that some substances decrease permeability while others increase it led the writer to the idea that substances of one class may antagonize those of the other.[2] It was of interest to test this idea by ascertaining whether sodium taurocholate can antagonize NaCl (which produces only an increase of permeability). Some experiments on this subject are described in the present paper.

The experiments were made by determining the electrical conductivity of *Laminaria*[3] in solutions to which sodium taurocholate was added.

In the first experiments the bile salt was dissolved in sea water. The amounts added to 1,000 cc. of sea water varied from 0.8 to 1.5 gm. If the sodium taurocholate were pure, 1 gm. in 1,000 cc. would make the concentration about 0.002 M, but as its purity is doubtful the concentration cannot be accurately determined.[4]

After dissolving the sodium taurocholate the sea water was restored to the normal conductivity and made approximately neutral to litmus.

---

[1] Cf. Osterhout, W. J. V., *Bot. Gaz.*, 1915, lix, 317, 364.

[2] Osterhout, W. J. V., *Science*, 1915, xli, 255.

[3] For the method see Osterhout, W. J. V., *J. Biol. Chem.*, 1918, xxxvi, 557.

[4] The salt used was the purest obtainable.

At all the concentrations employed there was an immediate increase in resistance[5] followed by a fall, as illustrated in Fig. 1. Under the conditions of the experiment (temperature 19° ± 2°C.) the rise lasted about an hour. The effect is comparable with that of anesthetics[6] (ether, chloroform, and alcohol) as described by the writer. An in-
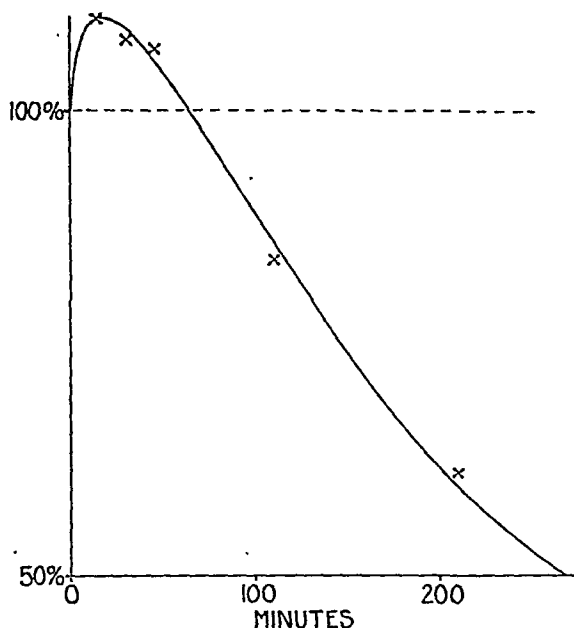
FIG. 1. Curve showing decrease of permeability (rise in net electrical resistance expressed as per cent of the control in sea water) of *Laminaria*, produced by adding 1 gm. of sodium taurocholate to 1,000 cc. of sea water (solid line). Control in sea water, dotted line. The resistance is expressed as per cent of the normal resistance in sea water, which is taken as 100 per cent. Average of two experiments; probable error less than 2.3 per cent.

crease in resistance was also observed with *Ulva rigida* and with *Rhodymenia palmata*.

In the experiments on antagonism the tissue was placed in a solution of NaCl 0.52 M to which various amounts of sodium taurocholate

[5] For convenience all the resistances are expressed as per cent of the normal resistance in sea water which is taken as 100 per cent.

[6] Cf. Osterhout, W. J. V., *Bot. Gaz.*, 1916, lxi, 148.

were added (all the solutions having the same conductivity as the sea water and being approximately neutral to litmus). The temperature was 18.5° ± 2.5°C.

The results are shown in Fig. 2. There is a gradual fall of resistance in all the solutions which continues until the death point (10 per
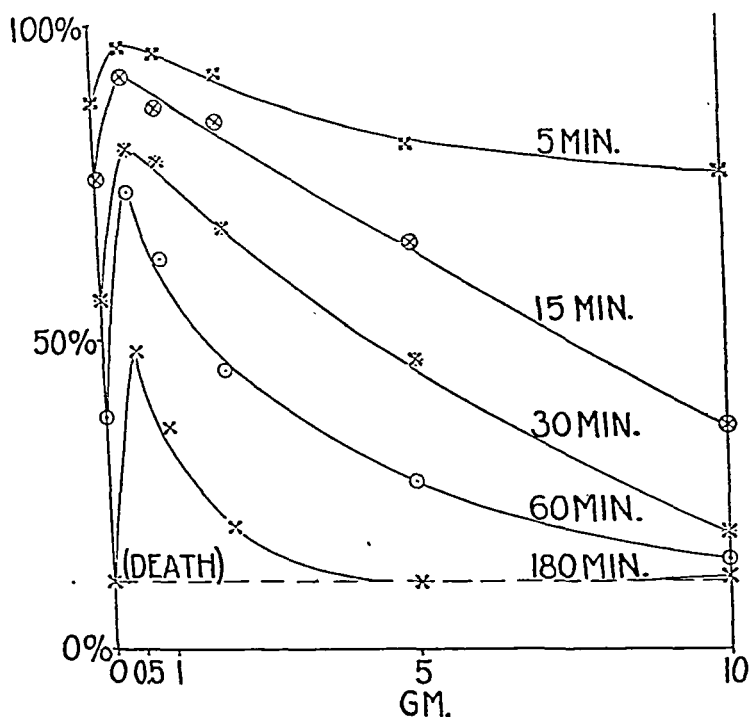
FIG. 2. Curves showing antagonism between NaCl and sodium taurocholate. The ordinates represent the net electrical resistance of *Laminaria* (expressed as per cent of the control in sea water which is taken as 100 per cent). The abscissæ represent the amount of sodium taurocholate added to 1,000 cc. of NaCl 0.52 M. Average of two experiments; probable error less than 5 per cent.

cent) is reached. In the solution containing 1,000 cc. of NaCl 0.52 M + 0.5 gm. of sodium taurocholate the fall of resistance is much slower, indicating that this is the most favorable mixture.

It should be emphasized that the effect is not an intermediate but an antagonistic one. By this is meant that the resistance is not

merely the algebraic mean between a rise in resistance produced by the bile salt and a fall produced by NaCl. A consideration of the lowest curve shows that at 180 minutes the tissue is dead in NaCl 0.52 M as well as in 1,000 cc. of NaCl 0.52 M + 10 gm. of sodium taurocholate, but in the mixture containing only 0.5 gm. of taurocholate it is not yet half dead, its resistance being much higher than in the other mixtures.[7]

The result serves as a striking confirmation of the idea that antagonistic relations can be predicted, to a considerable extent at least, by ascertaining the effect upon permeability of each substance taken by itself, inasmuch as substances which decrease permeability antagonize those which increase it.

SUMMARY.

Sodium taurocholate is able to produce a decrease in permeability and to antagonize NaCl. This confirms the hypothesis that antagonistic relations can be predicted from studies on the permeability of pure substances.

[7] At the end of 180 minutes the resistance of the control in sea water was 100 per cent.

A COMPARISON OF PERMEABILITY IN PLANT AND ANIMAL CELLS.

By W. J. V. OSTERHOUT.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

In a study of the fundamental characteristics of protoplasm it is desirable to compare the behavior of plants and animals under conditions which are as similar as possible. The experiments here described were undertaken from this point of view. Their object was to discover whether the behavior of animals and plants is essentially similar with regard to certain aspects of permeability, injury, recovery, and death.

The method consisted in measuring the electrical conductivity of the tissues. Under the conditions of the experiment the electrical conductivity of the tissue may be regarded as a measure of its permeability to ions.

The plant chiefly employed was Laminaria, although other material was used for comparison.[1] The tissue was cut into disks and packed together to form a cylinder whose electrical resistance was measured as previously described.[2]

The animal tissue used for comparison was the skin of the frog (Rana pipiens), taken from the animal immediately after killing[3] and placed at once in the apparatus for measuring electrical resistance. Inasmuch as the skin has not sufficient mechanical rigidity to permit the same kind of manipulation which is possible in Laminaria, it was necessary to fasten each piece between two thin hard rubber disks (or between a rubber disk and one of thin celluloid), the disks being tied together by means of projections at the edges and the space between

---

[1] Osterhout, W. J. V., J. Gen. Physiol., 1918–19, i, 299.
[2] Osterhout, W. J. V., J. Biol. Chem., 1918, xxxvi, 557.
[3] The animals were killed by decapitation. The use of anesthetics must be avoided as they may injure the skin.

409

them being partially filled with vaseline.[4]   The disks thus prepared were used in the same way as the disks of *Laminaria*.   The conditions were therefore strictly comparable.   In order to make this doubly sure, pieces of *Laminaria* of the same size as the pieces of frog skin were fastened between disks of hard rubber in the same manner and used for comparison.   The resistance of a pair of disks containing frog skin was in the neighborhood of three times that of a pair of disks containing *Laminaria*.   Usually five pairs of disks with frog skin were placed together to form a cylinder whose resistance was measured.[5]

The measurement of the resistance of the frog skin was less accurate than that of *Laminaria*.   The point of minimum sound in the telephone was more indefinite (especially with live tissue).   The resistance of the control was not constant as in the case of *Laminaria*. The controls of frog skin were placed in sea water + four volumes of distilled water, which was taken as approximately isotonic (for convenience this will be called 0.2 sea water).   In this solution the resistance sometimes remained constant for some time, but more often it rose somewhat and finally became constant for a time or else began slowly to fall.[6]   Increasing or diminishing the proportion of distilled water did not help.   Soaking the frog in the solution for an hour before removing the skin made no decided difference.   In spite of these difficulties it was possible to select from several lots of material some whose resistance did not change much in 0.2 sea water, and thus to obtain consistent results.   It was found desirable to express all the net resistances as per cent of the controls.[7]

[4] Cf. Osterhout, W. J. V., *J. Biol. Chem.*, 1918, xxxvi, 557.   The method shown in Fig. 7 was usually employed.   The apparatus described as type B was also used and gave similar results.

[5] Five pairs of disks containing *Laminaria* had about the same resistance as a cylinder of *Laminaria* tissue consisting of 80 pieces with only one rubber disk at each end.

[6] Regarding changes in the conductivity of frog skin, under various conditions, see Höber, R., Physikalische Chemie der Zelle und der Gewebe, Leipsic, 4th edition, 1915, 441.

[7] For example, a piece of frog skin was placed in NaCl and after a given time the resistance was measured; this was divided by the resistance of a control in 0.2 sea water measured at the same time (both lots having been placed in the solution at the same moment).   In 0.2 sea water the skin remained alive for several hours.

One of the most striking results observed in studying the electrical resistance of *Laminaria* is the fact that some substances (as CaCl₂) decrease permeability while others (as NaCl) increase it. . It is of interest to see whether animal tissue behaves in the same manner.

When *Laminaria* is placed in a solution of CaCl₂ (of the same conductivity as the sea water) the resistance[8] rapidly rises, often as much as 65 per cent; in LaNO₃ it rises even higher (90 per cent or more),[9] while in MgCl₂ the rise is less (up to 10 per cent). The rise is succeeded by a fall, which continues until death. The resistance has then dropped to about 10 per cent of the normal resistance.[10]

When frog skin is placed in CaCl₂ of the same conductivity as 0.2 sea water (about 0.056 M CaCl₂), the resistance rises rapidly, just as in the case of *Laminaria*, though not as high (up to 40 per cent). It then begins to fall, and continues to do so until the death point is reached[11] at about 10 per cent of the normal resistance. In LaNO₃ the rise is greater (up to 90 per cent), and in MgCl₂ it is less (up to 10 per cent). It will be seen that the behavior of frog skin toward these agents closely resembles that of *Laminaria*.

Acid (HCl) causes a rapid rise of resistance in *Laminaria*,[12] followed by a rapid fall. This is also the case with frog skin placed in a solution of HCl of the same conductivity as 0.2 sea water (about 0.024 M HCl) or in this solution diluted with 0.2 sea water. The use of HCl is attended by some complications which will be discussed in a later paper.

There is another group of substances, such as NaCl and KCl, which produce no rise in the resistance of *Laminaria*. On transferring tissue from sea water to solutions of these substances (of the same conductivity as sea water) there is a fall of resistance which continues until the death point is reached. The same is true of frog skin (using 0.2 sea water) except that the fall of resistance is much slower than in the case of *Laminaria*. It is, however, completely paralleled by the slow fall of resistance found in some other plant tissues, such as those of the dulse (*Rhodymenia palmata*).[1]

[8] Osterhout, W. J. V., *Bot. Gaz.*, 1915, lix, 317.

[9] Osterhout, W. J. V., *Bot. Gaz.*, 1915, lix, 464.

[10] The resistance of the apparatus is subtracted in all cases.

[11] The death point is not so well defined as with *Laminaria*.

[12] Cf. Osterhout, W. J. V., *J. Biol. Chem.*, 1914, xix, 493.

In mixtures of NaCl and CaCl$_2$ in suitable proportions[13] *Laminaria* remains alive longer than in pure NaCl or in pure CaCl$_2$. This is also true of frog skin, although the result is not as marked as in the case of *Laminaria;* this is perhaps due to the fact that NaCl is less toxic to frog skin.

Of special interest is the behavior of anesthetics. In the case of *Laminaria* they produce two effects, a reversible (anesthetic) action consisting in a decrease of permeability and an irreversible (toxic) action consisting in an increase of permeability.[14] On placing tissue in sea water containing suitable amounts of ether (1 per cent), chloroform (0.1 per cent), or chloral hydrate (0.1 per cent), the resistance rises and this condition is maintained for some time. With increasing concentration a point is soon reached at which the resistance rises rapidly to a maximum and then falls rather rapidly. When it has fallen below the normal there is little or no recovery on replacing it in sea water. With alcohol such recovery is possible.

The same is true of frog skin (using 0.2 sea water) but the effect is even more striking, the rise of resistance being greater and occurring at lower concentrations. In respect to recovery we find the same difference between ether, chloroform, and chloral hydrate on the one hand and alcohol on the other.

The method of measuring electrical resistance enables us to study the dynamics of the death process. It has been shown that when *Laminaria* dies in a solution of NaCl the process follows more or less closely the curve of a monomolecular reaction.[15] The same is true of frog skin. In both cases we are led to the assumption that the process of death is one which is always going on during the normal life of the cell and that it is accelerated by the toxic agent. It is also found, in both *Laminaria* and frog skin, that if the death process has not proceeded too far a complete or partial recovery is possible when the tissues are removed from the toxic solution and returned to sea water (or 0.2 sea water).

In both cases it appears that permeability is a delicate and accurate index of the vitality[16] of the protoplasm and that agents which pro-

[13] Osterhout, W. J. V., *Proc. Am. Phil. Soc.*, 1916, lv, 533.

[14] Osterhout, W. J. V., *Bot. Gaz.*, 1916, lxi, 148.

[15] Osterhout, W. J. V., *J. Biol. Chem.*, 1917, xxxi, 585.

[16] Osterhout, W. J. V., *Science*, 1914, xl, 488.

duce injury increase permeability. The amount of increase may be regarded as a measure of the amount of injury. A quantitative basis is thereby furnished for such conceptions as death, injury, recovery, and vitality.

The striking agreement between the behavior of frog skin and that of *Laminaria*, as well as of other plants previously studied,[1] strongly confirms the idea that the ideas which have been developed from the study of *Laminaria* are of general application. These ideas have been tested by the use of diverse methods.[17]

The general outcome of these studies reveals a marked amount of agreement, and it would seem that the physiological characteristics which they bring to light belong to the fundamental properties of protoplasm.

SUMMARY.

Quantitative studies show a striking agreement between frog skin and plant tissues in respect to certain important aspects of permeability, antagonism, injury, recovery, and death.

[17] Osterhout, W. J. V., *Science*, 1917, xlv, 97; *J. Biol. Chem.*, 1918, xxxvi, 485.
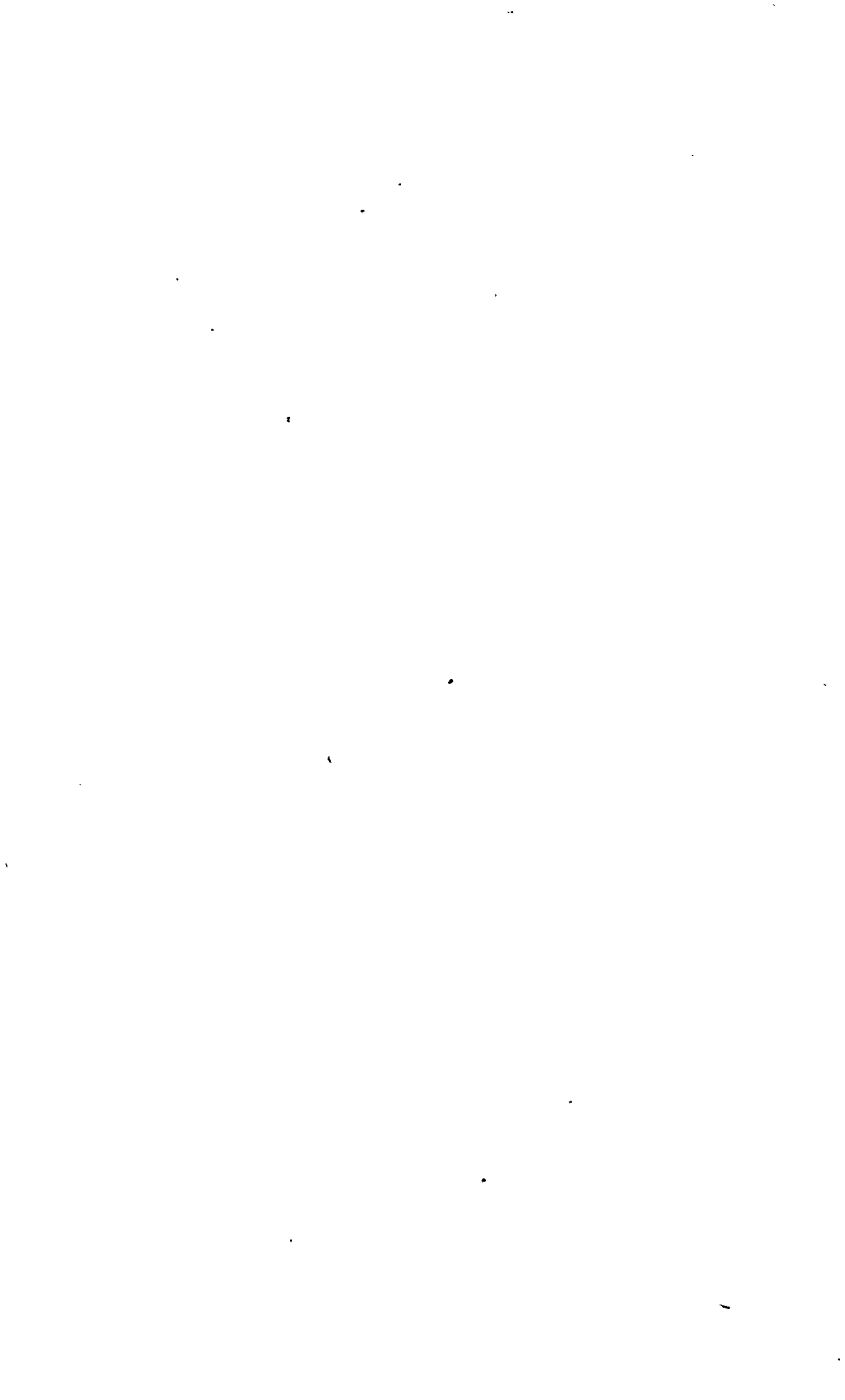
THE RELATION BETWEEN THE OXYGEN CONCENTRA-TION AND RATE OF REDUCTION OF METHYLENE BLUE BY MILK.

BY E. NEWTON HARVEY.

(*From the Physiological Laboratory, Princeton University, Princeton.*)

After the discovery of the reducing power of milk[1] and the proof that its reducing action on methylene blue (decolorization) is not due to bacteria but to a reducing enzyme,[2] Bach,[3] in a series of researches, undertook the study of reducing enzymes in various tissues. Schardinger[1] had originally found that fresh cow's milk will reduce methylene blue in the presence of an aldehyde but not in its absence and not if the milk had previously been boiled. In one paper Bach[4] showed that nitrates could also be reduced to nitrites by Schardinger's enzyme of milk and he studied the effect (on reduction) of nitrate, aldehyde, and enzyme concentration, as well as the influence of temperature. As the nitrate is still further reduced, the quantitative results obtained by determining the amount of nitrate formed at successive intervals of time after mixing varying quantities of nitrate, or aldehyde, or milk, are not of much value. They do show, however, that rate of nitrite formation increases with, but is not proportional to nitrate concentration or aldehyde concentration. Bach found also that nitrite formation is proportional to enzyme concentration, at least in the early stages of the reduction. He did not study the effect of oxygen concentration in milk on the reduction of nitrate.

[1] Schardinger, F., *Z. Untersuch. Nahrungs-u. Genussmittel*, 1902, v, 1113; *Chem. Ztng.*, 1904, xxviii, 704.

[2] Trommsdorff, R., *Centralbl. Bacteriol.*, *IIe Abt.*, *Orig.*, 1909, xlix, 291.

[3] Bach, A., *Biochem. Z.*, 1911, xxxi, 443; 1911, xxxiii, 282; 1912, xxxviii, 154; 1913, lii, 412.

[4] Bach, A., *Biochem. Z.*, 1911, xxxiii, 282.

This paper deals with the reduction of methylene blue by milk and acetaldehyde under varying partial pressures of oxygen. The study was undertaken through the observation that milk will reduce oxyluciferin to luciferin, the light-producing substance of luminous animals, or methylene blue to leucomethylene blue, only if the milk and reducible material stand for some time.[5] The reduction does not occur in either case if the mixtures are continually shaken with air. It appears that time is necessary for using up the dissolved oxygen present. If the oxygen is removed from a milk-acetaldehyde-methylene blue mixture by exhaustion with an air pump, decolorization (reduction) occurs almost immediately. Without exhaustion of the air such a mixture must remain undisturbed for over an hour at room temperature before reduction occurs.

Preliminary experiments showed that the amount of reducing enzyme varied in different samples of milk. The addition of 1 cc. M acetaldehyde to 10 cc. of one sample of milk gave the quickest reducing action. Either more or less aldehyde increased the time. Methylene blue was added till a blue color in the milk was marked (0.1 cc. 0.01 M (= 0.319 per cent) methylene blue was used). With less methylene blue slightly less time is required. The standard mixture was therefore 10 cc. of milk and 1 cc. M acetaldehyde + 0.1 cc. 0.01 M methylene blue. If shaken with air (21 per cent oxygen) and set aside in a test-tube at 20°C., the blue color in one particular case completely disappeared, except at the surface in contact with air, in 43 minutes. If shaken with air containing only half the normal amount of oxygen, decolorization occurs in 23 minutes, about one-half the time. The times for decolorization have been determined for other concentrations of oxygen and are given in Fig. 1 where times for decolorization are plotted along ordinates and oxygen concentrations along the abscissæ.

The reduction in concentration of oxygen was accomplished by partially exhausting the air over the milk-acetaldehyde-methylene blue mixture in a large test-tube with an air pump, at the same time shaking the mixture vigorously. Tubes which have been brought into equilibrium with air in partial vacua can be removed to air under

5 Harvey, E. N., *J. Gen. Physiol.*, 1918–19, i, 133.

atmospheric pressure and placed in a thermostat at 20° with safety because the diffusion of dissolved oxygen from the surface into the solution is very slow, if the tubes are undisturbed.
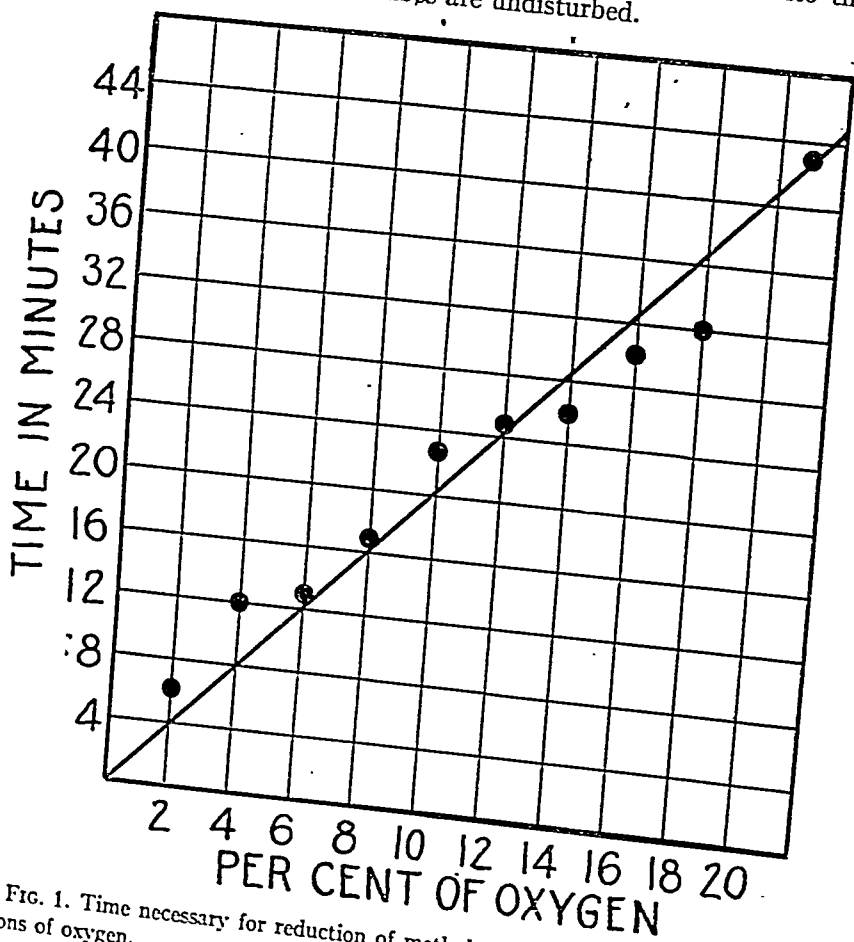
FIG. 1. Time necessary for reduction of methylene blue in varying concentrations of oxygen.

It will be noted from Fig. 1[e] that the curve is practically a straight line and the time for decolorization proportional to the oxygen concentration. It is obvious that this method can be used for determin-

[e] The results of this chart were obtained by Mr. J. P. Kelly, Princeton University, who undertook the determination at my suggestion.

ing the oxygen content in gaseous mixtures by use of the indicator methylene blue. The procedure differs somewhat from the indicator method recently described by Osterhout,[7] where organisms are placed in a hemocyanin solution (blue) and the time necessary for them to use up practically all of the oxygen is indicated by reduction to the colorless condition of the hemocyanin.

In using the milk-methylene blue method it is only necessary to shake a milk-acetaldehyde-methylene blue mixture with the gas to be analyzed and determine how long it takes for the blue color to disappear. The end-point can be quite accurately determined by comparison with a similar tube of milk containing no methylene blue. A control determination of the time necessary for decolorization of milk-acetaldehyde-methylene blue mixture shaken with air must be made under the same conditions. As air contains 21 per cent oxygen, if it takes 60 minutes to decolorize with air and 40 minutes to decolorize with the unknown gas, the latter must contain $\frac{40}{60}$ or $\frac{2}{3}$ of $21 = 14$ per cent of oxygen. Carbon dioxide in the gas up to 5 per cent does not affect the reducing action of milk.

The rate of decolorization of methylene blue by milk can be increased by raising the temperature or increasing the concentration of the reducing enzyme. This is easily done by evaporating the milk *in vacuo* to $\frac{1}{3}$ to $\frac{1}{4}$ its volume. The rate is roughly proportional to the concentration of the milk. Increase in temperature has the same marked accelerating action as on all chemical reactions, and it is important to maintain the temperature constant in all comparative work.

The reducing enzyme is unstable and cannot be preserved for any length of time by adding toluene, chloroform, or thymol to the milk. The addition of 2 per cent NaF to milk will prevent the growth of bacteria without affecting its reducing powers during a period of 2 months. Some samples of canned evaporated milk which I examined did not exhibit a reducing action. Since colloidal platinum and formic acid reduce methylene blue rapidly, imitating the milk-aldehyde reducing action,[8] it is likely that a protected platinum solution

[7] Osterhout, W. J. V., *J. Gen. Physiol.*, 1918–19, i, 167.

[8] Bredig, G., and Sommer, F., *Z. physik. Chem.*, 1910, lxx, 34.

might take the place of milk as a more stable medium for oxygen determinations.

SUMMARY.

The rate of reduction of methylene blue by milk and acetaldehyde is proportional to the concentration of oxygen in the milk. This fact may be made the basis of a method of determining oxygen in gaseous mixtures.

INFLUENCE OF TEMPERATURE AND HYDROGEN ION CONCENTRATION UPON THE SPORE CYCLE OF BACILLUS SUBTILIS.

By ARAO ITANO and JAMES NEILL

(*From the Department of Microbiology, Experiment Station, Massachusetts Agricultural College, Amherst.*)

The investigation here reported deals with the influence of hydrogen ion concentration at different temperatures upon the spore cycle of *Bacillus subtilis.*[1] This microorganism was chosen because it is a classical spore-bearing species, which had been used in our previous investigations on the relation of the hydrogen ion concentration of the medium to proteolytic activity.

TABLE I.

*Limiting Reactions.**

Organism.	pH	Author.
B. coli	5.0	Michaelis and Marcora.†
" " (low gas ratio group)	4.3–5.3	Clark, 1915.‡
Streptococcus, Group I	4.6–4.8	Ayers, 1916.§
" " II	5.5–6.0	" 1916.§
S. erysipelatis	4.8	Itano, 1916.‖
B. subtilis (acid limit)	4.2	" 1916.‖
" " (alkali limit)	9.4	" 1916.‖

* Clark, W. M., and Lubs, H. A., *J. Bacteriol.*, 1917, ii, 1, 222.

† Michaelis, L., and Marcora, F., *Immunitätsforsch., Orig.*, 1912, xiv, 170.

‡ Clark, W. M., *J. Biol. Chem.*, 1915, xxii, 87.

§ Ayers, S. H., *J. Bacteriol.*, 1916, i, 84.

‖ Itano, A., *Massachusetts Agric. Exp. Sta., Bull. 167*, 1916, 139.

---

[1] The same strain of this species was used as in the previous investigations on proteolytic activity; the strain was obtained from the American Museum of Natural History.

We have found no references in the literature to the particular problem involved, but it may be interesting to present in tabular form the limiting reactions of the medium for some bacteria.

Table I indicates that these organisms survive in a certain limited range of hydrogen ion concentrations, and that slight changes in the concentration of the hydrogen ion influence the organism to a great extent.

Method of Procedure.

Preparation of Media.—The media used in this investigation had the same composition and were prepared in the same way as those described in a previous article.[2]   The pH was determined electrometrically and was found to be as shown in Table II.

TABLE II.
Preparation of Media of Different pH Values.

pH desired..........	1	2	3	4	5	6	7	8	9	10	11	12	13
pH found...........	1.2	2.0	3.0	4.1	5.2	6.1	7.1	8.2	9.2	10.1	11.0	11.5	12.7

Throughout the following account, we have used the approximate figures in referring to the various pH values; *i.e.*, desired pH is used to denote the hydrogen ion concentration of the broth.

Method Used in Obtaining Free Spores.—The spores of the organism were obtained as follows: A portion of a young agar culture of *Bacillus subtilis* was emulsified in sterile 0.85 per cent salt solution. The emulsion was then transferred to a Roux flask containing standard agar, and the organisms were distributed over the surface of the medium.   The flask was incubated at 30°C. for 15 days and was then kept at 25°C. for 3 weeks.   At the time of the experiment the culture contained practically nothing but free spores.

Preparation of Moist Chambers.—Two moist chambers were made for each pH value for each temperature.   The chambers were prepared in the usual way, using paraffin of high melting point to seal the ring to the slide.   By means of a platinum loop 2 mm. in diameter,

[2] Itano, A., *Massachusetts Agric. Exp. Sta., Bull. 167*, 1916, 164.

a drop of medium of each pH value was put upon a sterile cover-slip which was then carefully sealed upon the ring of the moist chamber. The moist chamber preparations were then kept for 12 hours at the temperature to be used in the subsequent incubation.

Preparation of Dilute Emulsion of Spores.—Two drops of the medium of each pH value were put in the cells of sterile concave slides in sterile Petri dishes. With a straight needle a small inoculum of spores was put into the first drop of broth of each pH value. From this drop the second drop of the same pH value was then inoculated by use of the straight needle. The second drop was used as the source of the final inoculum.

Procedure in this manner with the medium of each pH value gave a very dilute emulsion of free spores in broth of each pH value. By this procedure we obtained for the inoculation of the hanging drops a satisfactory source for a small number of spores, well washed of all metabolic products; moreover, any of the medium carried over with the final inoculum was of the same pH value and of the same composition as the drop inoculated.

Inoculation of Drops.—The hanging drops of the moist chamber preparations were then inoculated; those of each pH value were inoculated from the dilute emulsion of spores in broth of the same pH value. By using a straight needle for transferring and by stirring the inoculated drop, a drop was obtained containing a satisfactory number of spores well distributed throughout the drop.

Examination of Preparations and Conditions of Observation.—Immediately after inoculation the preparations were placed under a microscope and examined for absence of vegetative forms and for even distribution of the spores. After examination the preparations were placed at the temperature of incubation. Observations were made at intervals of 30 minutes for the first 5 hours and then at intervals of 1 hour.

<div align="center">RESULTS.</div>

Series I (*5°C.*).—This series (pH 1 to 13) was incubated at 5°C. for 20 days.[3] No apparent change took place except a slight swelling of the spores in all hydrogen ion concentrations. This temperature

---

[3] The cold storage room of the Dairy Department of this institution was used.

seems to be near the limiting temperature for spore germination of *Bacillus subtilis*. Schreiber[4] reports the germination of spores of this organism at 8°C. after 7 days, but states that the spore cycle is not completed at this temperature.

Series II (25°C.).—This series was kept at room temperature, which varied during the time of the experiment from 23.5–25°C. The observations and results are given in Table III.

TABLE III.

Time in Hours Required by B. subtilis to Reach Various Stages of Development at Different pH Values. (*Temperature Approximately 25°C.*)

pH	Spore.		Vegetative cell.				Spore.	
	Swollen.	Germination begins.	Single.	Chains.	Granule.	Refractive body.	Endospore.	Free spore.
	hrs.	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*
1	S							
2	"							
3	"					·		
4	"							
5	2.5	7.5	10.0	12.0	25.0	30.0	32.0	50.0
6	2.5	6.0	8.0	9.0	22.0	30.0	31.0	46.0
7	2.0	4.0	5.0	6.0	20.0	26.0	29.0	39.0
8	2.0	3.0	4.0	5.0	16.0	41.0	51.0	65.0
9	3.0	7.0	10.0	13.0	20.0	44.0	52.0	66.0
10	3.5	8.0	11.0	15.0	22.0	25.0	31.0	39.0*
11	S							
12	"							
13	"							

Figures represent average of four experiments.

S indicates no apparent change except slight swelling and a certain loss of refrangibility.

* Few spores germinated; chains are short; not all cells formed spores.

The results in Table III show that at 25°C. the spores of *Bacillus subtilis* germinated in broth at pH values from 5 to 10, while higher and lower concentrations of hydrogen ions inhibited their development. At pH 7 and pH 8, which are nearest the optimum hydrogen ion concentration for this organism, germination took place in the shortest time and multiplication was most rapid.

[4] Schreiber, O., *Centralbl. Bakteriol., 1te Abt.*, 1896, xx, 432.

An interesting phenomenon was observed in the broth at pH 5, 6, and 9. At the time given in Table III only a few spores had germinated. These first vegetative cells did not exhibit the characteristic motility of this species, neither did rapid multiplication begin at once. Several hours later, however, after most of the spores had germinated the vegetative cells became actively motile and multiplication became much more rapid, indicating that by the life processes of the organisms

TABLE IV.

Time in Hours Required by B. subtilis to Reach Various Stages of Development at Different pH Values. (Temperature 37°C.)

pH	Spore.		Vegetative cell.				'Spore.	
	Swollen.	Germination begins.	Single.	Chains.	Granule.	Refractive body.	Endospore.	Free spore.
	hrs.	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*
1	S							
2	"							
3	"							
4	"							
5	2.0	11.0	13.0	15.0	20.0	24.0	27.0	32.0
6	1.5	8.0	9.0	10.0	14.0	19.0	22.0	28.0
7	1.5	5.0	7.0	9.0	12.0	17.0	19.0	26.0
8	1.5	3.5	6.0	8.0	10.0	12.0	13.0	20.0
9	1.5	4.0	5.0	7.0	8.0	10.0	14.0	23.0
10	1.5	11.0	12.0	14.0	15.0	18.0	25.0	35.0
11	S							
12	"							
13	"							

Figures represent average of four experiments.

S indicates no apparent change except slight swelling and a certain loss of refrangibility.

the reaction of the medium was approaching the optimum. In view of facts shown in a previous publication,[5] this behavior may be explained as a manifestation of the beginning of the automatic adjustment of the medium.

The behavior in pH 10 was also interesting. Only a few of the spores germinated and these passed into the spore stage in a comparatively short time without much further multiplication.

[5] Itano, A., *Massachusetts Agric. Exp. Sta., Bull. 167*, 1916, 174.

Series III (37°C.).—This series was kept in the incubator at 37°C.. The results are given in Table IV.

In general Table IV shows the same results as Table III, except that the rate of completion of the cycle was accelerated at the higher
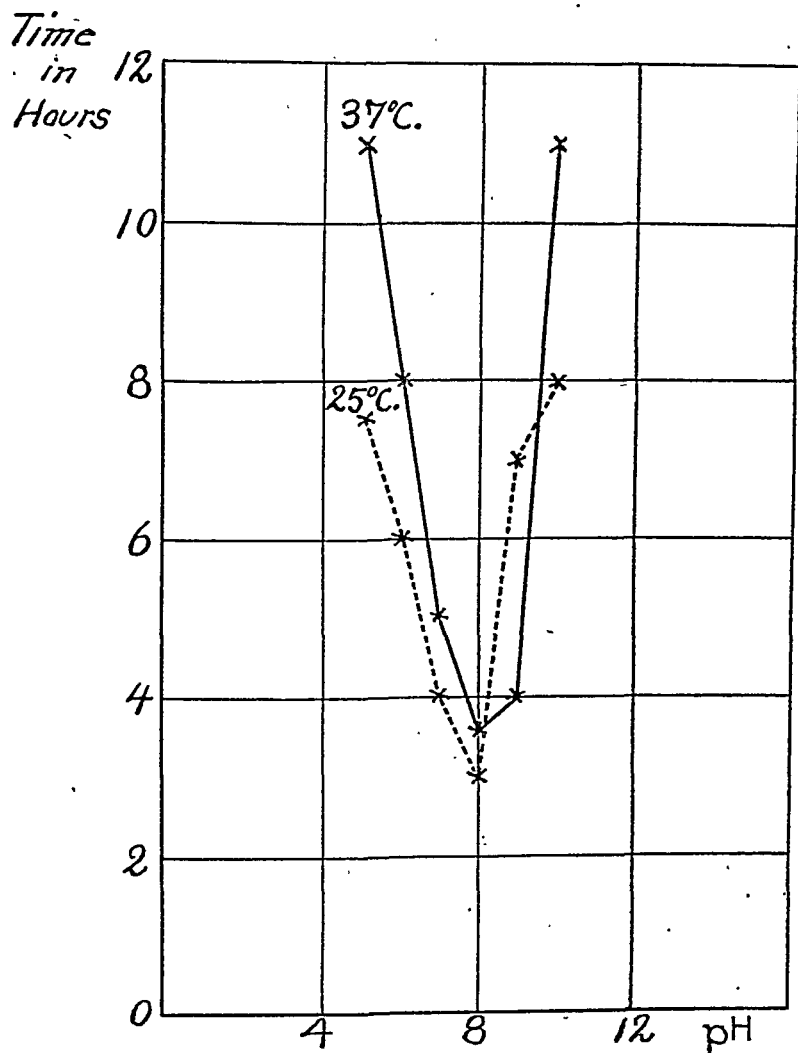
FIG. 1. Curves showing the time required for the germination of spores of *B. subtilis* in broth of different pH values at 25° and 37°C.
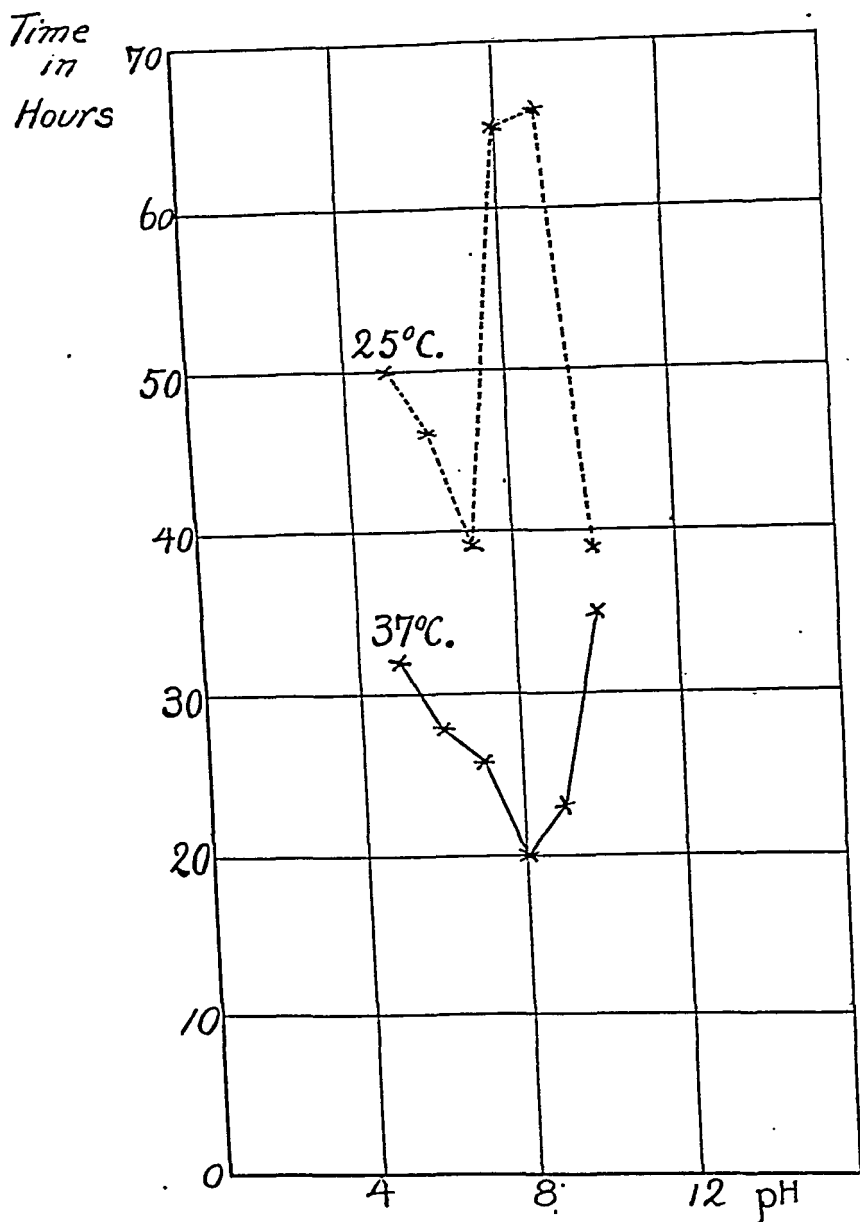
FIG. 2. Curves showing the time required for completion of the spore cycle of *B. subtilis* in broth of different pH values at 25° and 37°C.

temperature. The time required for germination does not show this acceleration, as is evident from Fig. 1. This indicates that 37° is decidedly above the optimum for germination.

The time required for the completion of the spore cycle is shown graphically in Fig. 2, which indicates that the spore cycle was completed only at pH 5 to 10 and that the rate of completion is accelerated by the rise in temperature. The acceleration, however, is irregular and does not maintain any uniformity throughout the series. The irregularity in the curve may be due to the previously mentioned automatic adjustment of the reaction of the media during the period of active growth which is included in the spore cycle. In broth of pH 5, 6, and 9, the hydrogen ion concentration would be gradually approaching the optimum during the period of active growth.

The apparent discrepancy in pH 10 of the 25°C. is probably due to the formation of spores because of the unfitness of medium of this pH value for growth and multiplication. The slight difference in time required for spore formation in broth of this pH value at 25° and 37° indicates that spore formation in this medium is probably not induced by products of metabolism.

SUMMARY AND CONCLUSIONS.

1. At 5°C. no germination took place.

2. At 25°C. and at 37°C. germination occurs if the hydrogen ion concentration of the broth is kept between pH 5 and pH 10, but not at higher or lower pH values.

3. The completion of the spore cycle likewise requires a hydrogen ion concentration between pH 5 and pH 10.

4. The spores can germinate when the pH value is 10, although after germination the vegetative cells multiply only to a very slight extent and soon pass into spores.

5. The slight growth and multiplication of vegetative cells in broth of pH 10 suggest that the formation of endospores in this medium must be caused largely by the unfavorable reaction of the medium rather than by the accumulation of metabolic products.

6. Automatic adjustment of the medium seems to play a rôle in the completion of the spore cycle.

7. The results are not only of theoretical importance but they have a practical application to the preservation of food by canning and by other methods.

BIOELEMENTS; THE CHEMICAL ELEMENTS OF LIVING MATTER.

By INGO W. D. HACKH.

(*From the College of Physicians and Surgeons, San Francisco.*)

Among the 87 known elements less than one-half (namely 34) have been found to enter into living matter. Of these only 17 seem to be essential to life, and of these 17 not more than 4 elements form from 97 to 99 per cent of the living organism.

In order to illustrate the importance of the different elements Fig. 1 and Tables I and II have been prepared, showing that some elements occur invariably, others frequently, and still others are of such rare occurrence that their presence in living matter seems adventitious.

In Fig. 1 the new periodic system[1] has been utilized to show that all the bioelements occupy neighboring places and that the majority of them possess low atomic weights, belonging to the first two periods of the system.

An approximation to the ultimate composition of man is given in Column 2 of Table I, while Column 3 shows the elementary composition of long leaf pine, calculated from the data of Little.[2] The chemical character of the mammal in comparison with the gymnosperm is evident, the latter containing 99 per cent of C, H, and O and only very small amounts of other elements, of which Al and Si are the predominant ones (due to the abundance of these two elements in the soil).

With the exception of oxygen, no element enters into the living organism as a free element, but only in the form of compounds, the majority of these being non-polar; that is, organic compounds. For

---

[1] Hackh, I. W. D., *J. Am. Chem. Soc.*, 1918, xl, 1023; *Am. J. Sc.*, 1918, xlvi, 481.

[2] Little, A. D., *J. Ind. and Eng. Chem.*, 1916, viii, 102.

FIG. 1. Bioelements and the periodic system. Bioelements invariably or frequently present in living organisms are indicated by a continuous arc; those which occur variably and rarely by a broken circle. The numbers indicate the order of their relative percentage. The elements of the lithosphere, hydrosphere, and atmosphere are also indicated by a continuous, broken, or dotted line. The elements occurring in meteorites are shown by a dot. The numbers indicate their relative abundance. This chart of the periodic system divides the elements into non-metals, light metals, and heavy metals. Missing elements are those of atomic numbers 43, 61, 75, 85, and 87, while Po = polonium, Ac = actinium, and Bv = brevium are radioactive substances, of which so far no long lived isotope is known. (See Hackh, I. W. D., *Am. J. Sc.*, 1918, xlvi, 481; *J. Am. Chem. Soc.*, 1908, xl, 1023.)

430

comparison the average composition of these compounds or foods is given in Columns 4, 5, 6, and 7 of Table I.

Regarding the distribution of the elements in the biosphere, there are the nine essential elements which are invariably present in the cell: C, H, O, N, P, S, Mg, Fe, and K, while eight more seem also to be important, for they are always present in small amounts in plant

<div align="center">TABLE I.</div>

Distribution of Bioelements.

Order.	Mammals.		Gymnosperms.		Average composition of foods.							
					Carbohydrates.		Fats.		Proteins.		Fresh water.	
(1)	(2) *per cent*		(3) *per cent*		(4) *per cent*		(5) *per cent*		(6) *per cent*		(7) *per cent*	
1	O	62.43	C	53.96	O	49.38	C	69.05	C	51.3	O .	88.80
2	C	21.15	O	38.65	C	44.44	O	17.90	O	22.4	H	11.16
3	H	9.86	H	7.13	H	6.18	H	10.00	N	17.8	S	0.018
4	N	3.10	Al	0.065	—		P	2.13	H	6.9	C	0.005
5	Ca	1.90	Si	0.057	—		N	0.61	S	0.8	Ca	0.004
6	P	0.95	S	0.052	—		S	0.31	P	0.7	Mg	0.003
7	K	0.23	Fe	0.030	—		—		Fe	0.1	N	0.002
8	S	0.16	N	0.030	—		—		—		Fe	0.002
9	Cl	0.08	Ca	0.007	—		—		—		P	0.001
10	Na	0.080	K	0.006	—		—		—		Na	0.001
11	Mg	0.027	P	0.005	—		—		—		K	0.001
12	I	0.014	Mg	0.003	—		—		—		Remainder	0.003
13	F	0.009	Cl	0.002	—		—		—		—	
14	Fe	0.005	Na	0.001	—		—		—		—	
15	Br	0.002	F	0.001	—		—		—		—	
16	Al	0.001	Mn	0.001	—		—		—		—	
17	Si	0.001		—	—		—		—		—	
18	Mn	0.001		—	—		—		—		—	
		100.00		100 00		100.00		100.00		100.00		100.00

Column 2 represents, in per cent, the elementary composition of man; Columns 4, 5, 6, and 7 give the average composition of common foods. The data of Column 3 are calculated from the results of Little and represent the elementary composition of the long leaf pine.

and animal organisms; namely, F, Cl, Br, I, Si, Na, Ca, and Mn. These seventeen bioelements are shown in their relative order in Fig. 1, all of them occupying neighboring places.

In addition to these elements there are some apparently essential to certain species of plants and animals, such as Al, Cu, Cs, B, Ba,

TABLE II.

Distribution of Chemical Elements.

Order.	Lithosphere.		Hydrosphere.		Atmosphere.		Meteorites.	
(1)	(2) *per cent*		(3) *per cent*		(4) *per cent*		(5) *per cent*	
1	O	47.33	O	85.79	N	75.53	Fe	72.06
2	Si	27.74	H	10.67	O	23.02	O	10.10
3	Al	7.85	Cl	2.07	Ar	·1.40	Ni	6.50
4	Fe	4.50	Na	1.14	H	0.02	Si	5.20
5	Ca	3.47	Mg	0.14	C	0.01	Mg	3.80
6	Na	2.46	Ca	0.05	Kr	0.01	S	0.49
7	K	2.46	S	0.05	Xe	0.005	Ca	0.46
8	Mg	2.24	K	0.04	Remainder	0.005	Co	0.44
9	Ti	0.46	N	0.02	—		Al	0.39
10	H	0.22	Br	0.01	—		Na	0.17
11	C	0.19	C	0.01	—		P	0.14
12	P	0.12	I	0.006	—		Cr	0.09
13	S	0.12	Fe	·0.002	—		C	0.04
14	Mn	0.08	Remainder	0.002	—		K	0.04
15	Ba	0.08	—		—		Mn	0.03
16	F	0.07	— ·		—		Ti	0.01
17	Cl	0.06	—		—		Cu	0.01
18	N	0.02	—		—		Remainder	0.03
19	Sr	0.02	—		—		—	
	Remainder	0.51	—		—		—	
		100.00		100.00		100.00		100.00

The data of Columns 2, 3, and 4 are taken from Clarke,[4] those of Column 5 from Farrington, O. C., (*Field Museum Nat. History, Publication 120,* 1907; *Publication 151,* 1911), whose report contains analyses of 318 iron and 125 stone meteorites.

Li, Rb, and Zn, while traces of As, Ce, Co, Cr, Mo, Ni, Pb, Ra, Sr, and Ti, have also been reported in specific instances. These elements are marked by a broken circle in Fig. 1 and are also situated close together, with two exceptions.[3]

This summary would be incomplete without a consideration of the abundance of the elements upon our earth. In Table II the data calculated by Clarke[4] are systematically arranged and the order of occurrence in the three spheres (gaseous, liquid, and solid) has been embodied in Fig. 1. It is significant that only the four elements C, N, O, and H occur in all three spheres, while only seven, S, Cl, K, Na, Mg, Ca, and Fe, are predominant in two spheres. Thus the elements of living matter are also the common elements of the earth surface.

Plant organisms contain relatively more of the elements of the soil. Titanium seems to be an exception; although it occurs in traces in nearly all plants,[5] we know little of its physiological functions. Its abundance in celestial bodies, revealed by the spectrum of titanium oxide, would indicate a more important rôle of this element than is known at present.

In conclusion, only 5 per cent of the known elements (namely, C, N, O, and H) forms from 96 to 99 per cent of the biosphere,[6] while about 15 per cent of the elements is invariably or frequently present, another 20 per cent sometimes occurs in traces in certain organisms, and the remaining 60 per cent of the elements is absent under normal conditions; thus from 60 to 80 per cent of the elements (absent and rarely encountered) are of a more or less poisonous character.

[3] These two exceptions, Ce and Pb, would indicate that other members of the carbon group, Zr and Sn, should be found in organisms.

[4] Clarke, F. W., *U. S. Geol. Survey, Bull. 616*, 3rd edition, 1916.

[5] Robinson, W. O., Steinkoenig, L. A., and Miller, C. F., *U. S. Dept. Agric., Bureau of Soils, Bull. 600*, 1917.

[6] These four elements have also been utilized for a system of structure symbols of organic compounds (see Hackh, I. W. D., *Canadian Chem. J.*, 1918, ii, 135; *Science*, 1918, xlviii, '333).
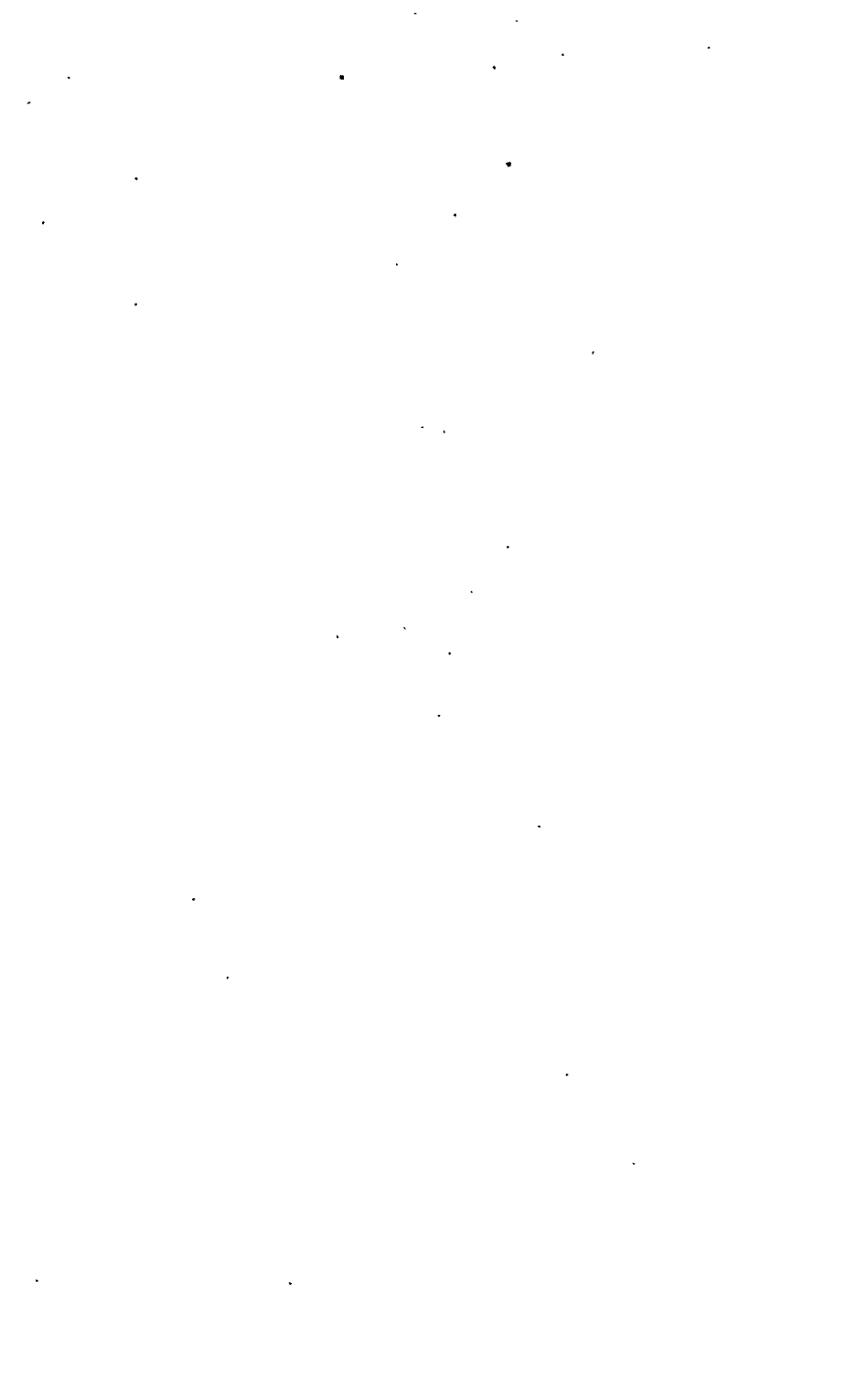
PHOTOREACTIONS OF PARTIALLY BLINDED WHIP-TAIL SCORPIONS.

By BRADLEY M. PATTEN.

(*From the Laboratory of Histology and Embryology, School of Medicine, Western Reserve University, Cleveland.*)

INTRODUCTION.

The experiments dealt with in this paper were devised to ascertain (1) the relative effectiveness as photoreceptors of the whip-tail scorpion's median eyes, lateral eye groups, and cutaneous sensitive areas, and (2) the effect on orientation produced by symmetrical and by asymmetrical interference with the photoreceptive mechanism.

Most of the animals familiar in the literature on phototropism have light-sensitive mechanisms which consist of a single pair of receptors, or mechanisms in which one of the receptors overshadows the others in effectiveness. The whip-tail scorpion has three pairs of photoreceptors, each of which, acting alone, is capable of bringing about orientation. It offers, therefore, unusually varied possibilities for experiments altering the normal, symmetrical condition of the photoreceptive mechanism.

As a basis for working on partially blinded scorpions, the responses of normal animals were quantitatively determined. The results of this preliminary work have already been published (1), but certain of the more important points may be summarized here. The species of whip-tail used (*Mastigoproctus giganteus* Lucas) was found to be negatively phototropic, and very consistent in its precision of orientation. The method of measuring the normal reactions and the experimental conditions under which the measurements were carried out, were chosen with a view to making even slight changes in reaction clearly recognizable. Responses to known intensities of illumination

435

were recorded in terms of deflection from an initial direction of loco-
motion. Deflection amplitudes were measured in degrees by record-
ing the animal's point of emergence from a graduated circle. By
making the diameter of the circle relatively small (50 cm.), the rate of
attaining orientation, as well as the accuracy of orientation, was made
measureable.

The accuracy of orientation exhibited by normal animals when
placed between equal, opposed beams of light, was measured as the
reaction which would be most readily disturbed by any asymmetrical
interference with the receptive mechanism. Deflection amplitudes of
animals subjected to anterior and to lateral illumination were meas-
ured as being the reactions which would be most extensively changed by
symmetrical eliminations of receptors.

The experiments on partially blinded animals reported below were
carried out with the same intensity of illumination, and the same
method of handling used on normal animals. Change from the nor-
mal reaction following the elimination of a photoreceptor can, there-
fore, be taken as a measurable index of the effectiveness of the receptor
prevented from functioning. A series of experiments in which all
possible cases of elimination are carried out will show at the same
time the relative effectiveness of the different members of the receptive
system, and the effect on orientation produced by symmetrical and
by asymmetrical interference with the photoreceptors.

Apparatus.

The apparatus used in these experiments was the same as that used
in measuring the reactions of normal animals. It is shown in Fig. 1.

Determinations of the illumination delivered by the lights were
made with a Bunsen photometer. Each fixed light gave an illumina-
tion of 120 candle meters at the center of the observation circle. The
intensity of the third light could be varied by moving it to different
positions along the axis. The whole apparatus was located in a dark
room and in order to reduce reflected light to a minimum, all parts of
it except the scales were painted flat black.

Methods.

The general external anatomy of the whip-tail scorpion and the more important points concerning its experimental handling have been taken up in a preliminary paper (1). For a detailed morphological consideration of the group to which *Mastigoproctus giganteus*
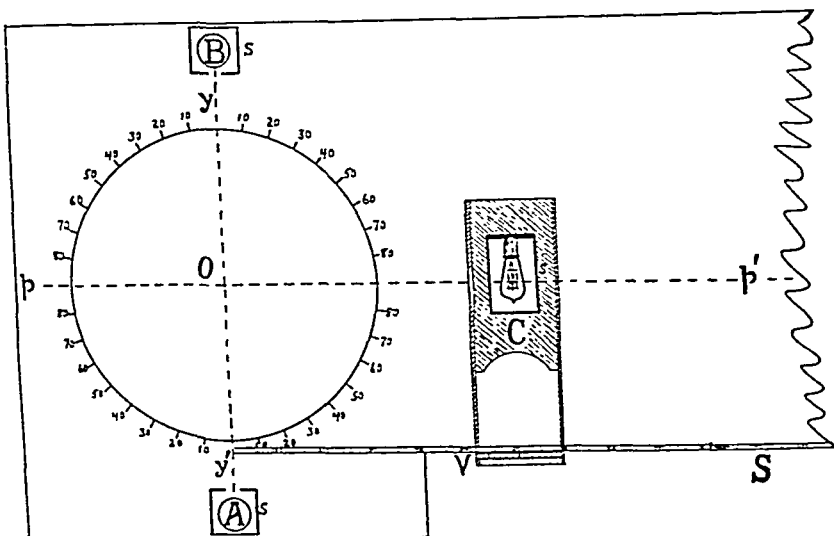
FIG. 1. Plan of apparatus. About O as a center a circle 50 cm. in diameter was described and graduated in degrees. This is termed the "observation circle". The axis pp' is constructed parallel to the edge of the table and the axis yy' perpendicular to pp'. On the axis yy' two 15 watt Mazda lamps, A and B, were set up, each distant 35 cm. from the center of the circle. On the axis pp' a similar lamp was mounted on a movable frame, C. Affixed at right angles to the base of the frame, C, was a scale, V, reading as a vernier against the scale, S, at the edge of the table. Light-proof cases, s,s,s, enclosed each light except for diaphragms 3×3 cm. so placed that the beams of light transmitted each centered at O.

belongs reference should be made to Börner (2). The sketch of *Mastigoproctus* reproduced in Fig. 2 will serve to give the location of the photoreceptors considered in this paper.

As has already been stated, the method mapped out for securing data on the effectiveness of the various photoreceptors consisted in comparing with the reactions of normal animals, the reactions of ani-

mals in which parts of the light-sensitive system had been prevented from functioning. Various means of eliminating the lateral and median eyes were considered. There seemed no justification, in the
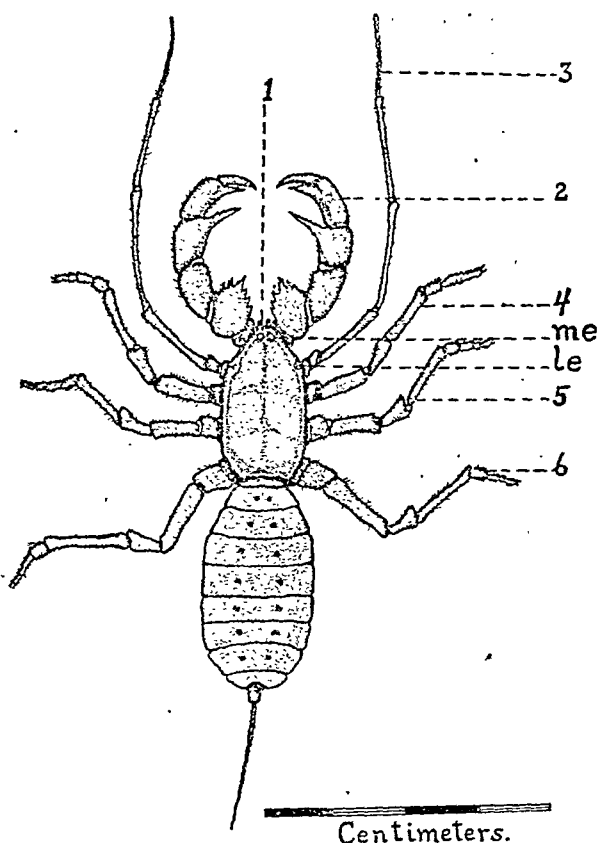
FIG. 2. *Mastigoproctus giganteus* Lucas. *1*, chelicerae; *2*, pedipalps; *3*, modified anterior legs which serve as feelers; *4, 5, 6*, walking legs; *me*, median eyes; *le*, lateral eye group consisting of three eyelets clustered very close together in the form of a miniature triangle.

case of an animal in which the eyes could be effectively covered, for such violent methods as extirpation or cauterization with their possible disturbing after-effects. The following two methods of covering

the eyes were both found to be satisfactory and one or the other was used, without preference, in the series of experiments.

1. Asphaltum varnish was applied with a camel's hair pencil to the surface of the eye and allowed to dry until sticky. A cap of tin-foil cut to fit over the eye was then set in the varnish and allowed to dry in hard. Finally another coat of asphaltum was put over the cap, especial care being used to seal the edges tightly.

2. Rubber adhesive tape was given two coats of asphaltum varnish on the fabric surface. When the second coat had dried, the tape was cut into caps of the desired size, which were pressed firmly over the eyes. The caps were then sealed on with asphaltum varnish and allowed to dry.

Subsequent microscopical examination of cleaned exoskeletons of animals in which the eyes had been capped in these ways gave no indication of light leakage. Furthermore, either type of covering could be removed, leaving the eye uninjured for control experiments.

The first animals handled after the capping of both median eyes and both lateral eye groups showed still a well defined sensitiveness to light. After careful rechecking of the work for possible light leakage around the caps showed that the eyes were effectively covered, a systematic search was begun for cutaneous photosensitive areas.[1] Animals were placed under vertical illumination of just sufficient intensity to make their outlines discernible and searched with a point of intense light obtained by placing a tungsten flashlight bulb in place of the ocular of a compound microscope and converging the rays through the objective (3). As might be expected, the areas of the body covered by thick, heavily pigmented chitin were insensitive to light. This narrowed the search to two localities: the "feeling legs" (see Fig. 2) which had already been demonstrated to be sensitive to touch

---

[1] The possibility that blinded scorpions might be reacting to the heat of the light was tested in the following way. Animals were illuminated from one side by light of 120 candle meters. On the opposite side, 20 cm. distant from the scorpion, was placed a flatiron emanating heat rays plainly discernible to the back of the hand. The light drove both normal animals and animals with their median and lateral eyes capped toward the iron until they burned their feelers on it. In spite of their crudeness these experiments served to rule out the possibility of a heat reaction playing any part under the conditions of these experiments.

and to chemicals, and the areas of extremely thin and unpigmented chitin around the articulations of the appendages with the sides of the cephalothorax. Careful searching of the feelers with the light spot failed to elicit any reaction from the animal or movement of the feelers. Furthermore when the feelers were cut off several animals, some of which were otherwise normal and some of which had both median and lateral eyes capped, the characteristic orientation to light was in neither case affected.

Attention was then turned to the areas of thin chitin at the sides of the cephalothorax. Searching these areas with the light spot caused the animals to turn away from the illuminated side. The same areas were then rendered opaque by painting with asphaltum varnish.[2] Animals with one side of the cephalothorax blackened, when subjected to bilaterally balanced illumination, showed a very marked deflection toward the blackened side. This deflection was not attributable to mechanical interference with locomotion due to the presence of the varnish, for when stimulated mechanically, the animals did not move in curves. In another series of cases, scorpions with all eyes capped and both sides of the cephalothorax blackened were found to be insensitive to light. Methylene blue preparations of the integument in this region made subsequently showed abundant ganglion cells and nerve endings.

These observations indicated that the analysis of the receptive mechanism must deal with three elements: the median eyes, the lateral eye groups, and the photosensitive areas at the sides of the cephalothorax where the legs articulate with the body. With effective methods of preventing any receptor or group of receptors from functioning, the work resolved itself into series of measurements in which all possible combinations of interference with the photoreceptors should be covered.

---

[2] The results of this treatment were at first somewhat unsatisfactory because of the mucus thrown out when the areas in question were irritated by the varnish. It was possible, however, by repeated paintings to get a nearly unbroken coat of varnish to adhere. Animals treated in this way had to be used in the desired experiments before the varnish had dried long enough to become brittle.

Reaction Measurements.

In all the tests with partially blinded animals, great care was used to reproduce the experimental conditions under which the corresponding tests on normal animals had been made. The method of handling the animals and the methods of measuring and tabulating the reactions were the same as those described for normal animals (1). The details have, therefore, been omitted and the data presented only in the form of tabular and graphic summaries.

For convenience in presentation and consideration, the reaction measurements have been collected in three groups: (1) The reactions to balanced opposed illumination of animals subjected to asymmetrical interference with the photoreceptive mechanism. (2) The reactions to balanced opposed illumination of animals subjected to symmetrical interference with the photoreceptive mechanism. (3) The reactions to lateral and to anterior illumination of animals subjected to symmetrical interference with the photoreceptive mechanism.

Reactions to Balanced Opposed Illumination of Animals Subjected to Asymmetrical Interference with the Photoreceptive Mechanism.

In all the experiments on asymmetrical interference, the blackening was carried out on the right side in one-half of the animals, and on the left side in the other half of the individuals used. Tables I and II show the results of measurements made on animals with one lateral eye group capped, and on animals with one side of the cephalothorax blackened. These tables are given in detail partly as illustrations of the method of handling the measurements, but especially to show the consistency and the range of individual variability encountered in typical series of measurements. Qualitatively there can be no doubt as to the significance of the reactions. The approximate consistency of the individual reactions would indicate that averages obtained from the ten trials of an animal express with reasonable quantitative accuracy the value of the reaction.

In the case of the other series of measurements detailed tables have been omitted and only the summary of the results presented. Table III summarizes the measurements made under balanced illumination, on animals subjected to asymmetrical interference with the photoreceptors.

TABLE I.

Reactions to Balanced Illumination of Animals with Lateral Eye Group on One Side of Head Covered. Measurements Recorded in Degrees of Deflection from Initial Path of Locomotion Perpendicular to Line Connecting Two Sources of Light.

No. of trial.	Animal 6. Right lateral eye group capped.		Animal 10. Right lateral eye group capped.		Animal 8. Left lateral eye group capped.		Animal 9. Left lateral eye group capped.	
	Deflection to right.	Deflection to left.	Deflection to right.	Deflection to left.	Deflection to right.	Deflection to left.	Deflection to right.	Deflection to left.
	degrees	*degrees*	*degrees*	*degrees*	*degrees*	*degrees*	*degrees*	*degrees*
1	30		35			22		20
2	50		22			32·	0	0
3	28		20			28		40
4	38		45			30		30
5	40		25			62 ·		30
6	50		0	0		33		20
7	46		22		5			10
8	20		50			42		20
9	22		10			38	0	0
10	40		30			45		20
Total...	364		259		5	332		190
Average.	36.4 to right.		25.9 to right.		32.7 to left.		19 to left.	

TABLE II.

Reactions to Balanced Illumination of Animals with Cutaneous Photosensitive Areas on One Side of Body Blackened. Measurements Recorded in Degrees of Deflection from Initial Path of Locomotion Perpendicular to Line Connecting Two Sources of Light.

No. of trial	Animal 21. Cutaneous sensitive areas on left side blackened.		Animal 22. Cutaneous sensitive areas on left side blackened.		Animal 23. Cutaneous sensitive areas on right side blackened.		Animal 24. Cutaneous sensitive areas on right side blackened.	
	Deflection to right.	Deflection to left.	Deflection to right.	Deflection to left.	Deflection to right.	Deflection to left.	Deflection to right.	Deflection to left.
	degrees	*degrees*	*degrees*	*degrees*	*degrees*	*degrees*	*degrees*	*degrees*
1		12		52	30		30.	
2		26		30	· 12		20	
3		12		24	40		56	
4		13		25	50		24	
5		30		22	21		34	
6		28		42	52		60	
7		36		12	30		20	
8		50		18	22		20	
9		48		16	28		16	
10		31		10 ·	34		24	
Total...	·	286		251	319		304	
Average.	28.6 to left.		25.1 to left.		31.9 to right.		30.4 to right.	

TABLE III.

Summary of Measurements on Asymmetrically Blinded Animals under Balanced Illumination.

Operation.	Deflections from initial path of locomotion perpendicular to the line connecting two equally intense sources of light at its mid-point.				
	Individual averages each based on ten trials.				Average deflection toward less sensitive side.
	Animals with right side less sensitive. Deflections to right.		Animals with left side less sensitive. Deflections to left.		
	Animal 1.	Animal 2.	Animal 1.	Animal 2.	
	degrees	*degrees*	*degrees*	*degrees*	*degrees*
One median eye capped.	10.5	14.1	8.2	29.7	15.6
Both lateral eye groups and median eye on one side capped.	23.0	20.8	17.5	10.3	17.9
Both cutaneous sensitive areas and both lateral eyes blackened and median eye on one side capped.	21.4	31.3	23.0	32.9	27.1
One lateral eye group capped.	36.4	25.9	19.0	32.7	28.5
Cutaneous sensitive areas on one side blackened.	31.9	30.4	28.6	25.1	29.0
Both lateral eye groups and both median eyes capped and cutaneous sensitive areas of one side blackened.	37.5	35.5	37.2	34.0	36.0
One median eye and one lateral eye on same side capped.	35.7	42.3	36.6	·35.9	37.6
One median eye and the cutaneous sensitive areas on the same side blackened.	35.4	33.3	37.4	41.6	36.9
One lateral eye and the cutaneous sensitive areas on the same side blackened.	41.0	48.8	46.5	39.2	43.9
All photoreceptors on one side·blackened.	Circus movements.	Circus movements.	Circus movements.	Circus movements.	Circus movements.

The measurements given in Tables I to III are graphically summarized in Fig. 3. The circles represent the observation circle of the apparatus, and the groups of arrows the direction of the lights. The initial position of the animal is indicated by the outline in the center of the circle, and the average path of an animal, based on ten trials, by the radial arrows. On the same figure the average path of travel followed by normal animals, under the same experimental conditions, is indicated by shading. The arrows showing deflections to the right and to the left indicate the courses of animals with the blackening carried out on their right and on their left sides respectively.

In every one of the ten combinations of eliminations in which the photoreceptive mechanism was left functionally asymmetrical, the deflection appears toward the side made less sensitive.

Reactions to Balanced Opposed Illumination of Animals Subjected to Symmetrical Interference with the Receptive Mechanism.

As controls to the experiments in which the receptive mechanism was rendered functionally asymmetrical, measurements were made covering the reactions to balanced illumination of animals which had been subjected to bilaterally symmetrical eliminations of their light-sensitive organs.

The measurements are summarized in Table IV and represented graphically in Fig. 4. The manner of representation is the same as that in Fig. 3, the shaded area representing the reaction range of normal animals, and the radial arrows the average paths of the experimental animals. In none of the cases do the reactions of animals with the photosensitive mechanism left functionally symmetrical vary appreciably from the reactions of normal animals. This series of measurements stands in striking contrast to that of Fig. 3 with which it should be compared.

Reactions to Lateral and to Anterior Illumination of Animals Subjected to Symmetrical Interference with the Photoreceptive Mechanism.

While symmetrically blinded animals showed little or no variation from the normal when subjected to balanced illumination, under lateral or anterior illumination their attainment of orientation was retarded.
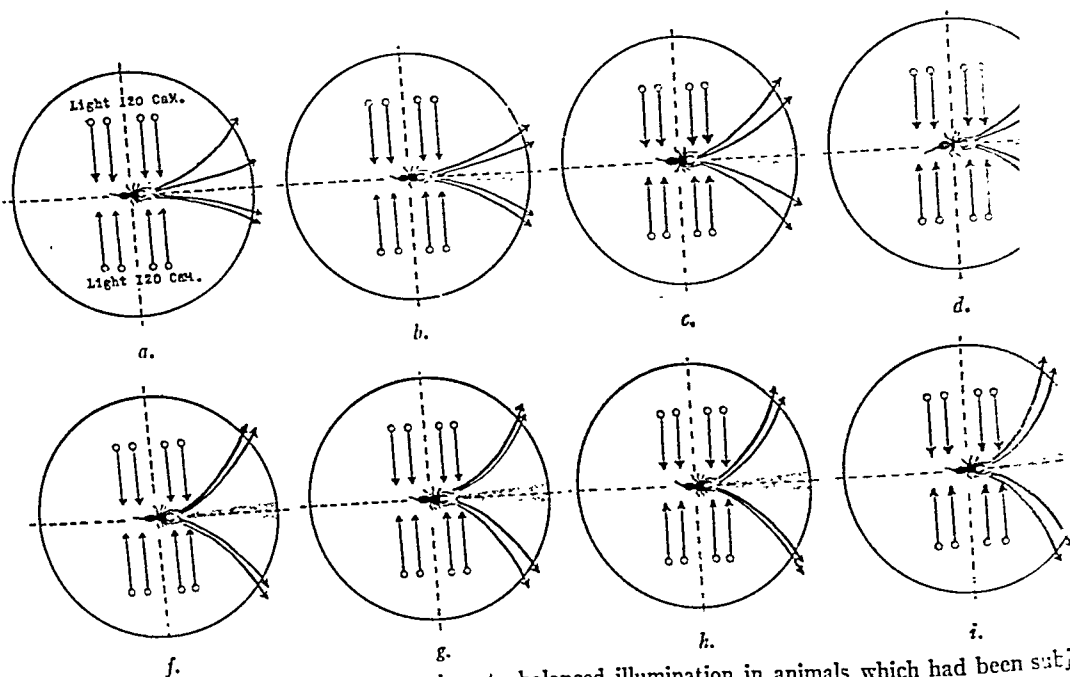
FIG. 3. Graphical summary of reactions to balanced illumination in animals which had been subje... ence with their photoreceptive mechanism. The circle represents the observation circle of the appar... direction of the light beams; the silhouette of the scorpion, the animal's position when subjected to ill... segment, the average reaction range of normal animals; the curved radial arrows, the average paths of... on ten trials). *a*. One median eye capped. *b*. One median eye and both lateral eye groups capp... lateral eyes, and both cutaneous sensitive areas blackened. *d*. One lateral eye group capped. *e*. Cu... side blackened. *f*. Cutaneous sensitive area of one side blackened and median and lateral eyes on both... and one lateral eye on the same side capped. *h*. One median eye capped and the cutaneous sensitive... ened. *i*. One lateral eye group capped and the cutaneous sensitive area of the same side blackened... side of the body blackened.

This difference in response in the two cases is due to the difference in the way the animals were brought into the field of light. When subjected to balanced illumination, their initial direction of locomotion was "in orientation" and it was only necessary to maintain it. The symmetrically reduced photosensitive mechanism did this nearly as effectively as the normal. Under lateral or anterior illumination the

TABLE IV.

Reactions to Balanced Illumination of Animals Previously Subjected to Symmetrical Interference with Photoreceptive Mechanism.

Operation.	Deflection from initial path of locomotion perpendicular to line connecting two equally intense sources of light at its mid-point.				
	Individual averages based on ten trials.				Average path of all animals tested.
	Animal 1.	Animal 2.	Animal 3.	Animal 4.	
	degrees	*degrees*	*degrees*	*degrees*	*degrees*
Both median eyes capped.	0.3 to right.	0.8 to left.	0.1 to left.	0.3 to right.	0.1 to left.
Both lateral eyes capped.	2.3 to left.	2.9 to left.	3.5 to right.	1.6 to right.	0.03 to left.
Cutaneous sensitive areas on both sides blackened.	0.2 to left.	3.9 to right.	4.3 to right.	0.6 to right.	2.1 to right.
Both median and both lateral eyes capped.	4.2 to right.	4.7 to left.	3.7 to right.	0.2 to right.	0.9 to right.
Both lateral eyes capped and cutaneous sensitive areas on both sides blackened.	2.1 to right.	4.7 to right.	4.9 to right.	3.0 to left.	2.2 to right.

animal must *change its direction of locomotion to come into orientation*, and this was accomplished less rapidly in animals with reduced photosensitive areas than in normal individuals. Doubtless if the final orientation were measured, it would vary but little from that of normal animals. Since, however, the reactions are measured by point of emergence from a standard circle; even slight variations in the rate of coming into orientation became apparent, because the method

was based on the degree of orientation attained while the animal was traveling over a standard distance, thus taking into account the time factor.

Fig. 4. Graphical summary of reactions to balanced illumination in animals which had been subjected to symmetrical interference with their photoreceptive mechanism. The circle represents the observation circle of the apparatus; the groups of arrows, the direction of the light beams; the silhouette of the scorpion, the animal's position when subjected to bilateral illumination; the shaded segment, the average path of normal animals; the curved radial arrows, the average paths of experimental animals (based on ten trials). a. Animals with both median eyes capped. b. Both lateral eye groups capped. c. Cutaneous sensitive areas on both sides blackened. d. Both lateral and both median eyes capped. e. Both lateral eyes and the cutaneous sensitive areas on both sides blackened.

The reaction measurements made on symmetrically blinded animals subjected to lateral illumination are collected in Table V and in Fig. 5. The solid arrows represent the average path followed by the partially blinded animals, the dotted arrows the average reaction of normal animals under the same conditions of illumination.

TABLE V.

Reactions to Lateral Illumination of Animals Previously Subjected to Symmetrical Interference with Photoreceptive Mechanism.

Operation.	Deflection from initial path of locomotion at right angles across the beam of light.				
	Individual averages based on ten trials.				Average of all animals tested.
	Animal 1.	Animal 2.	Animal 3.	Animal 4.	
	degrees	*degrees*	*degrees*	*degrees*	*degrees*
Both median eyes capped.	61.3	61.9	58.3	55.2	59.2
Both lateral eyes capped.	43.5	50.5	60.3	67.4	55.4
Cutaneous sensitive areas of both sides blackened.	60.1	48.1	38.9	45.1	48.1
Both median and both lateral eyes capped.	52.5	41.3	39.5	44.2	44.4
Both lateral eyes capped and cutaneous sensitive areas of both sides blackened.	42.6	30.4	44.6	23.8	35.4
All photoreceptors blackened.	+8.8*	6.3	4.6	2.0	1.0

The plus sign indicates that this deflection was *toward* the light.   All other deflections in the table are away from the light, or negative.

TABLE VI.

Reactions to Anterior Illumination of Animals Previously Subjected to Symmetrical Interference with Photoreceptive Mechanism.

Operation.	Deflection from initial path of locomotion directly toward source of light.				
	Individual averages based on ten trials.				Average of all animals tested.
	Animal 1.	Animal 2.	Animal 3.	Animal 4	
	degrees	*degrees*	*degrees*	*degrees*	*degrees*
Both median eyes capped.	117.3	123.5	136.6	132.6	127.5
Both lateral eyes capped.	124 9	128.8	127 8	140.9	130.6
Cutaneous sensitive areas of both sides blackened.	120 2	121.0	101.2	100.4	110.7
Both lateral and both median eyes capped.	118.3	116 0	108.3	115.8	114.6
Both lateral eyes capped and cutaneous sensitive areas on both sides blackened.	91 5	88.8	98.4	101.0	94.9

Table VI and Fig. 6 show the reactions of symmetrically blinded animals to anterior illumination. The experimental conditions under which these measurements were made are essentially similar to those
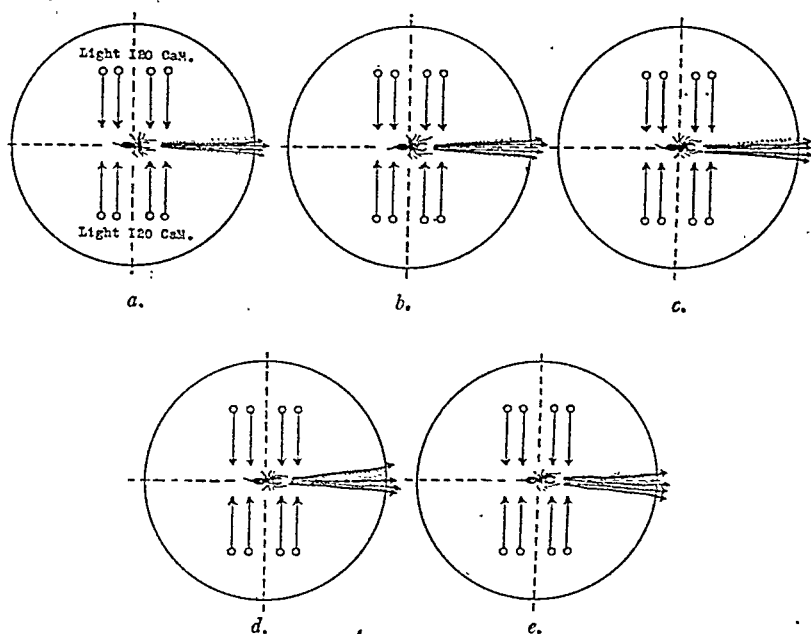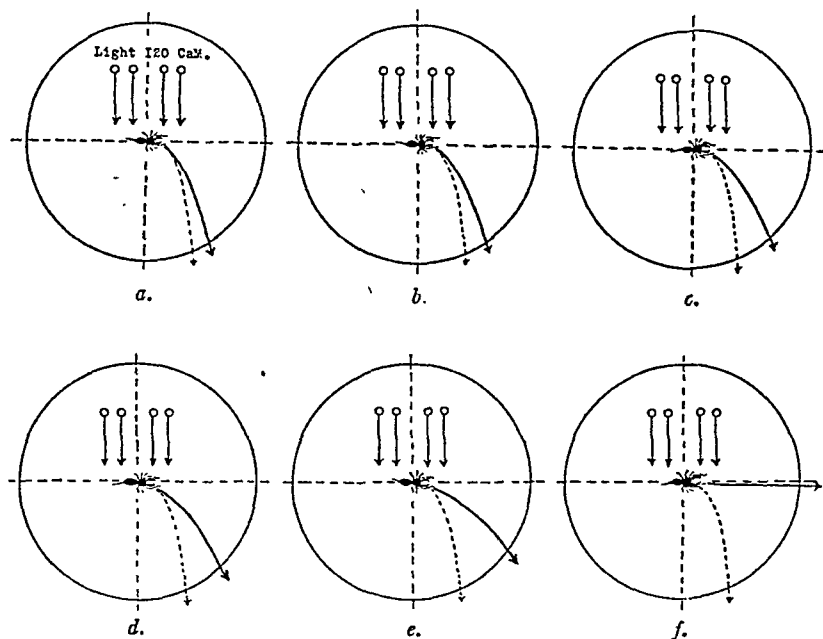

FIG. 5. Reactions to lateral illumination in animals which had been subjected to symmetrical interference with their photoreceptive mechanism. The circle represents the observation circle of the apparatus; the group of arrows, the direction of the light beam; the silhouette of the scorpion, the animal's position when subjected to lateral illumination; the dotted arrow, the average reaction of normal animals; the solid arrow, the average reaction of the experimental animals. *a.* Animals with both median eyes capped. *b.* Animals with both lateral eye groups capped. *c.* Cutaneous sensitive areas on both sides blackened. *d.* Both median and both lateral eyes capped. *e.* Both cutaneous sensitive areas and both lateral eyes blackened. *f.* All photoreceptors blackened.

set up when lateral illumination was used. The difference lies only in the greater deflection an animal must make to come into orientation when subjected to anterior illumination. A comparison of Figs.

5 and 6 shows that the reactions under lateral and under anterior illumination are qualitatively the same.

Quantitative comparison based on the percentage reduction of the normal reaction in each case shows a correspondence which is
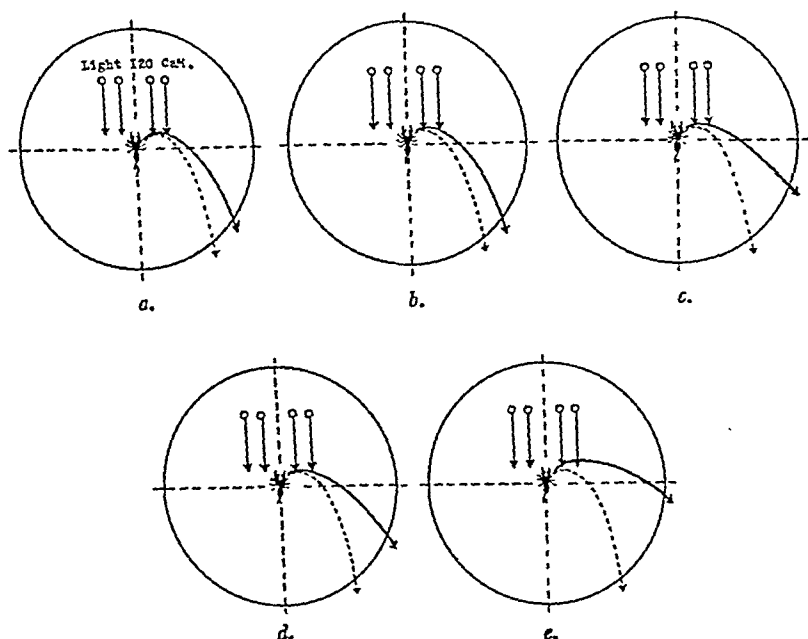
FIG. 6. Reactions to anterior illumination in animals which had been subjected to symmetrical interference with their photoreceptive mechanism. The circle represents the observation circle of the apparatus; the group of arrows, the direction of the light beam; the silhouette of the scorpion, the animal's position when subjected to anterior illumination; the dotted arrow, the average reaction of normal animals; the solid arrow, the average reaction of the experimental animals. a. Animals with both median eyes capped. b. Animals with both lateral eyes capped. c. Cutaneous sensitive areas on both sides blackened. d. Both median and both lateral eyes capped. e. Both cutaneous sensitive areas and both lateral eyes blackened.

within the limits of experimental error, when the differences in effective illumination due to the anatomical position of the receptors is taken into consideration (see below).

DISCUSSION.

Relative Effectiveness of Photoreceptors.

The relative effectiveness of the lateral eye groups, the median eyes, and the cutaneous photosensitive areas should be indicated by the changes from the normal reactions produced by their elimination. Table VII is a summary of the data secured by unilateral and bilateral elimination of each of the photoreceptors. The change from the normal reaction is given (1) in degrees of deflection; (2) in per cent

TABLE VII.

Summary of Effects of Eliminating Different Members of Photoreceptive Mechanism.

Organ eliminated.	Unbalance as measured under bilateral illumination.		Pair of organs eliminated.	Reduction from normal reaction as measured under lateral illumination.			Reduction from normal reaction as measured under anterior illumination.		
	Deflection toward blackened side.	Relative values of deflections toward the blackened side.		Measured in degrees of deflection.	In per cent of normal reac-tion.	Relative values.	Measured in degrees of deflection.	In per cent of normal reac-tion.	Relative values.
	degrees								
One median eye.	15.6	1.0	Both median eyes.	6.6	10	1.0	12.9	9.2	1.0
One lateral eye.	28.5	1.8	Both lateral eyes.	10.4	15.9	1.6	9.8	7.0	0.8
One cutane-ous sensi-tive area.	29.0	1.9	Both cutane-ous sensi-tive areas.	17.7	26.8	2.6	29.7	21.2	2.3

of the normal reaction; and (3) in relative values calculated by taking the change produced by elimination of the median eyes as unity.

There are certain apparent inconsistencies in the data of Table VII that require consideration before we attempt to draw any conclusions as to the relative effectiveness of the photoreceptors.

There is good agreement between the reductions in normal reaction produced by the elimination of the median eyes under lateral and under anterior illumination, the value of the reduction from normal being 10 per cent under lateral illumination and 9.2 per cent under

anterior illumination. The changes from the normal reaction induced by elimination of the lateral eyes, however, do not show the same consistency, the values being 15.9 per cent reduction of the normal reaction under lateral illumination, and 7 per cent reduction under anterior illumination. This discrepancy is, I believe, attributable to the anatomical location of the lateral eyes. Their position at the sides of the cephalothorax, surrounded by heavily pigmented chitin, is such that a very small proportion of the light in the field would be effective on the retinulæ of the lateral eyes when the animal is directly facing the light. The lateral eyes would, therefore, be operating below their capacity during a considerable part of the deflection made by animals headed into the light. On the other hand, they would be operating at maximum efficiency during the greater part of the deflection made by animals subjected to lateral illumination. The median eyes are so placed that they receive approximately the same amount of effective illumination whether the animal is subjected to anterior or to lateral illumination. The conditions of shading, involved in the initial positions of the animal account, therefore, for the discrepancy in the two sets of reaction measurements made on animals with their lateral eyes covered. The same considerations would indicate that measurements under lateral illumination express more correctly than experiments under anterior illumination the relative effectiveness of the photoreceptors.

Another apparent inconsistency in the data of Table VII is revealed by adding the reductions in deflection produced by the separate elimination of each receptor. Taking as a basis the measurements made under lateral illumination, the reductions in deflection caused by the separate elimination of the three photoreceptors total to a value approximately only one-half the normal reaction; yet when the three receptors are simultaneously eliminated the animals are practically insensitive to light (Table V and Fig. 5). It is possible to attribute these conditions to a compensatory increase in activity on the part of the unblackened receptors. While this may be in part responsible, I believe the greater part, at least, of the discrepancy can be otherwise explained. In each of the series of measurements in which some part of the photoreceptive mechanism is prevented from functioning, the reduction in deflection is measured at what might be called the

upper end of the deflection range. It has been shown (1) that the increase in illumination necessary to produce an initial deflection of definite value is less than the increase in illumination necessary to produce an increase in deflection of like value. The data collected were not sufficiently extensive to justify the statement that the increases in deflection followed mathematically the Weber-Fechner law, but the curve of increase in deflection with increasing lateral illumination is certainly of that general type. Applied to the data under consideration, this means that the first 10° of deflection are more readily induced than the deflection from 10° to 20° and so on. A reduction in deflection to 50° from a normal of 60°, following interference with receptors, does not indicate therefore that the receptive mechanism has been reduced by one-sixth in efficiency. On the contrary, it would require a reduction of considerably more than one-sixth in photoreceptive effectiveness to reduce by one-sixth the normal reaction.

While it is not possible, in the light of the above considerations, to compute the effectiveness of the different receptors on the basis of the percentage reduction of normal reactions produced by their elimination, their relative effectiveness may, nevertheless, be deduced from the available data. All the induced reductions in reaction, because of the method of measurement employed, fall in the same part of the deflection range. They can, therefore, justifiably be compared with each other. This comparison has been worked out in Table VII by taking the effect of the elimination of the median eyes as unity and comparing with it the effect produced by elimination of the other receptors. By averaging[3] the values thus obtained under the three different conditions of illumination used, we can approximate the relative effectiveness of the photoreceptors as median eyes : lateral eyes : cutaneous areas :: 1 : 1.6 : 2.2.

[3] In computing the averages the responses to anterior illumination made by animals with their lateral eyes capped were not given equal weight with those made under lateral illumination. The reasons for regarding the experiments under anterior illumination as less accurate as far as indicating the effectiveness of the lateral eyes is concerned, are given in the text.

Effect on Orientation Produced by Symmetrical and by Asymmetrical Interference with the Photoreceptive Mechanism.

Small changes in the tonus of the muscles of locomotion are frequently difficult to detect and always difficult to handle quantitatively in an animal which is not moving. An animal's locomotion, however, is a direct resultant of muscular activities and any difference in the tonus or in the vigor of contraction of the muscles on opposite sides of the body is at once evidenced by a deflection in the path of advance. Small differences in contraction are summated in continued locomotion. The measurement of deflections in the path of travel is, therefore, a convenient and accurate method of dealing quantitatively with the activities of locomotor muscles.

Unbalanced muscular reactions as indicated by a curved path of locomotion have been observed to follow unilateral blinding in both positively and negatively phototropic animals. The earlier observations were qualitative only, more recently the reactions have been quantitatively handled. In all the cases the evidence is in accord with Loeb's muscle tension theory of orientation, positively phototropic forms moving in curves with their uninjured side inward, and negative forms in curves with their blackened side inward (4).

The reaction measurements made in terms of angular deflection on partially blinded whip-tail scorpions fall entirely in line with observations on other animals. But because the scorpion has three pairs of photoreceptors, each of which can be eliminated separately, the series of measurements which can be made on it is peculiarly extensive and interesting. It is possible to eliminate on one side of the body the median eye, the lateral eye, or the cutaneous sensitive areas; or any two receptors; or all three receptors. By making eliminations on both sides of the body, the series of cases may be still further extended. In these experiments measurements were made, under bilaterally balanced illumination, on animals in ten different conditions of asymmetry. The results are collected in Table III. The graphical summary (Fig. 3) shows clearly the unerring consistency with which all the animals were deflected toward their less sensitive side. All the deflections are to be regarded as having the same significance as circus movements. In most of the deflections the radius of the curved

path followed by the animals is greater than the radius of the observa-
tion circle of the apparatus. When the induced asymmetry is ex-
treme, typical circus movements of small radius result. The point of
emergence of the trails of larger radius on the standard observation
circle, gives an index of the curvature of the arc of locomotion in
quantitative terms which are comparable with the other reaction meas-
urements. The constant curving of the path of locomotion in ani-
mals which are asymmetrically sensitive stands in sharp contrast to
the locomotion of normal animals under bilaterally unequal illumina-
tion. In the latter case the path of locomotion is straight once the
direction of crawling becomes such that the inequality in illumination
in the field is equalized in its effectiveness on the photosensitive areas
of the animal by greater exposure of the sensitive areas on the side of
less intense illumination, and lesser exposure of the sensitive areas on
the side of more intense illumination (5). Asymmetrically sensitive
animals under vertical illumination continue in a curving path
of locomotion because it is impossible through changes in axial posi-
tion to equalize bilaterally the effective illumination. Under equal
and opposed horizontal illumination slight bilateral inequalities in
sensitiveness may be compensated for by the assumption of an axial
position inclined toward the less intense light. When the asymmetry
of sensitiveness is made extreme the effective illumination cannot thus
be brought into bilateral equilibrium and the curved path of locomo-
tion is continued.

A feature deserving further comment is the way in which the am-
plitude of the deflections increases as the degree of asymmetry in
sensitiveness is increased. The cumulative effect produced by black-
ening more than one receptor on the same side of the head is obvious
from the figures. Less apparent but equally significant is the fact
that the unbalance in reaction is greater when all the receptors except
a given one are eliminated, than when the same receptor is the only
one covered. For example, the deflection induced when the median
eye on one side is capped averages 15.6°; when both lateral eyes, the
cutaneous sensitive areas on both sides, and the median eye on one
side are blackened (leaving functional only one median eye), the de-
flection averages 27.1°(see Table III). The unbalanced factor in each
experiment is a median eye, but when the median eye alone is elim-

inated the balance of the receptive mechanism as a whole is less disturbed than when the median eye on one side is the only functional receptor.

It is not pertinent here to enter into a detailed comparison of these results on the scorpion with the experiments of Garrey (6) on the positively phototropic robber fly, but the complete agreement of the two series of observations made by different methods, on animals having opposite signs of phototropism, is too striking to pass without comment.

Further evidence that balanced reactions depend on the functional symmetry of the receptive mechanism is furnished by the measurements made on animals in which the photoreceptors had been symmetrically interfered with. As in the case of asymmetrically sensitive scorpions, the symmetrically blinded animals were brought into the field of equal opposed lights "in orientation" (*i.e.*, with their plane of symmetry perpendicular to the line connecting the sources of light). No matter what eliminations were made, so long as the photoreceptive mechanism was left in a functionally symmetrical condition it sufficed to maintain[4] a balanced response which was accurate within the limits of variability exhibited by normal animals under the same conditions of illumination (Table IV and Fig. 4).

Observations already published by many different investigators have covered nearly every phase of the correlation existing between bilaterally balanced excitation of photoreceptors and balanced locomotor responses. A constantly increasing accumulation of experimental evidence indicates that the attaining and maintaining of orientation to light depends, as postulated in Loeb's muscle tension theory, on the transmission to the muscles of locomotion of impulses which are proportional bilaterally to the excitation of symmetrically located photoreceptors.

The results of the experiments described above indicate that the muscle tension theory applies to the complex receptive mechanism of

[4] It has already been pointed out that when brought into the field of light out of orientation (as in the experiments under anterior and lateral illumination) symmetrically blinded animals exhibited a retardation in their rate of coming to a new direction of orientation.

the whip-tail scorpion as well as to animals with a single pair of receptors. The evidence may be summarized as follows:

In normal scorpions bilateral equilibrium in excitation results invariably in bilaterally balanced muscular responses and a straight path of locomotion.

When the receptive mechanism is reduced but left functionally symmetrical, the locomotor responses remain balanced. Animals in five different symmetrical conditions of reduced sensitiveness showed no unbalance in reaction although the rate of attaining orientation was reduced in proportion to the extent of the interference with the photoreceptive mechanism.

When the receptive system is subjected to eliminations which leave it in an asymmetrical condition, unbalanced locomotor responses invariably follow, resulting in a curved path of locomotion with the less sensitive side of the animal on the inside of the curve. In a series of ten different conditions of asymmetrical sensitiveness the degree of unbalance in locomotion was proportional to the extent of asymmetry which had been produced in the receptive apparatus.

In their effect on orientation, the three pairs of receptors are completely coordinated, the excitation of the organs on the same side of the head being summated in transmission to the associated muscles of locomotion.

Balanced muscular reactions with a persistently straight path of locomotion depend on bringing the excitation of the receptive mechanism functional at the time into bilateral equilibrium.

SUMMARY.

The experiments dealt with in this paper were devised to ascertain (1) the relative effectiveness as photoreceptors of the whip-tail scorpion's median eyes, lateral eye groups, and cutaneous sensitive areas, and (2) the effect on orientation produced by symmetrical and by asymmetrical interference with the photoreceptive mechanism.

Each of the receptors was eliminated unilaterally and bilaterally, singly and in combinations with other receptors. In all, sixteen different abnormal conditions of the photoreceptive apparatus were produced.

The reactions of animals thus partially blinded were measured in terms of angular deflection from an initial path of locomotion. Measurements obtained under anterior, lateral, and bilaterally balanced illumination were compared with measurements made on normal animals under the same conditions of illumination. The change from the normal reaction induced by covering a photoreceptor was taken as an index of the effectiveness of the receptor prevented from functioning.

By comparing the values of the changes from normal reactions produced by the elimination of the several receptors, their relative effectiveness is approximated as median eyes : lateral eyes : cutaneous sensitive areas :: 1 : 1.6 : 2.2.

All animals in which the receptive mechanism was rendered functionally asymmetrical exhibited, when subjected to bilaterally balanced illumination, deflections toward the side which had been made less sensitive. In a series of measurements made on animals in ten different conditions of asymmetry the amplitudes of the deflections were proportional to the degree of unbalance which had been produced in the photosensitive mechanism.

Animals in which the receptive mechanism was reduced but left in a symmetrical condition maintained an undisturbed balance of reaction when subjected to equal, opposed lights. Under lateral or anterior illumination the rate of attaining a new direction of orientation was reduced in proportion to the extent of the interference with the receptive mechanism.

The reactions of symmetrically and asymmetrically blinded scorpions indicate that orientation is attained and maintained by a transmission of impulses to the muscles of locomotion which is proportional bilaterally to the excitation of the symmetrically located photoreceptors.

In their effect on orientation the three pairs of receptors are completely coordinated. Orientation depends upon bringing the excitation of the receptive mechanism as a whole into bilateral equilibrium.

BIBLIOGRAPHY.

1. Patten, B. M., *J. Exp. Zool.*, 1917, xxiii. 251.
2. Börner, C., *Zoologica*, 1904, xvii. No. 42. 1-174.
3. Patten, B. M., *Science*, 1915, xli. 141.

4. Loeb, J., Forced movements, tropisms, and animal conduct, Philadelphia and London, 1918.
5. Patten, B. M., *J. Exp. Zool.*, 1914, xvii, 213.
6. Garrey, W. E., *Proc. Nat. Acad. Sc.*, 1917, iii, 602; *J. Gen. Physiol.*, 1918–19, i, 101.

A STUDY OF THE ACTION OF ACID AND ALKALI ON GLUTEN.

By L. J. HENDERSON, EDWIN J. COHN,* P. H. CATHCART,*
J. D. WACHMAN,* and W. O. FENN.*

*(From the Wolcott Gibbs Memorial Laboratory, Harvard University, Cambridge, in
Collaboration with the Division of Food and Nutrition, Medical
Department, U. S. Army.)*

The problem of the acid-base equilibrium in systems containing protein substances and electrolytes has often been obscured by superficial interpretations of experimental results and, in recent investigations, by undue emphasis upon the colloidal aspects of the phenomena. Nevertheless, it has always remained certain that proteins, as amphoteric substances, must under all circumstances, except when pure at the isoelectric point, combine chemically with acids or bases. Moreover, the simple relationships involved in such equilibria are the most important factors in determining the properties of the systems. This conception of the behavior of protein systems has recently been reaffirmed and amplified by Sörensen and his associates,[1] and by Loeb.[2]

The studies which are reported in the present paper point in this same direction. Our experiments have been made with gluten, in some respects not the most favorable material for investigation, because it contains two distinct proteins, because it has not been highly purified from electrolytes, and because it possesses marked colloidal properties. In spite of these obstacles, which must therefore be less important than they seem, the results indicate a simple chemical interpretation of the phenomena.

This research was undertaken under the pressure of war time practical interests, for the purpose of explaining the technology of bread

* Lieutenant, Sanitary Corps, U. S. Army.

[1] Sörensen and others, Compt. rend. trav. Lab. Carlsberg, 1917, xii.
[2] Loeb, J., J. Biol. Chem., 1917, xxxi, 343; 1918, xxxiii, 531; 1918, xxxiv, 77, 395, 489; 1918, xxxv, 497; J. Gen. Physiol., 1918–19, i, 39.

making. Accordingly the measurements have in some respects not been pushed to the highest possible accuracy, nor has every part of the question been carefully surveyed.

I.

The first part of the work consisted of measurements, with the concentration cell, of the hydrogen ion concentration of solutions which had been in contact with powdered gluten.

The data in Table I show that, as a first approximation, the hydrogen ion concentration in such systems is determined by the ratio of gluten to acid, or gluten to base. In the acid systems this is quite clear; in the alkaline systems, where unknown difficulties have interfered with the accuracy of the measurements, it is perhaps somewhat less apparent. But, in view of the fact that under the circumstances equilibrium can hardly be perfectly attained, and on account of the numerous other difficulties which are inherent in work of this kind, the evidence seems satisfactory.

This result accords with the work of Sörensen. But the system that we have studied contains large amounts of protein in a discrete phase, and frequently also large amounts dispersed throughout the aqueous solution, and therefore possesses a very high degree of complexity. It is on this account especially important that the simple relationship still holds good. Unpublished measurements upon purified glutenin and gliadin further confirm this observation. Therefore we venture to draw the conclusion that in systems containing gluten and acids or bases the formation of salts, in accordance with the requirements of the mass law, is the fundamental phenomenon. Here, as in all cases where a weak acid or base in excess is in equilibrium with a strong base or acid, the hydrogen ion concentration is dependent upon the ratio of acid to salt or of salt to base, as the case may be. This relationship is of course only approximate, less true in the neighborhood of the isoelectric point. Compared with the case of simple substances, it is modified in a definite and characteristic manner by the polybasic and polyacid character of the amphoteric protein. It is not, however, seriously modified by the heterogenity of the system or by the colloidal character of the protein. This subject will be discussed theoretically in a later paper.

TABLE I.

Hydrogen Ion Concentration of Solutions of NaOH and HCl containing Gluten in Different Concentrations.

A

Gluten in 100 cc. solution.	Concentration of HCl.	$\left(R \dfrac{HCl}{gluten}\right)$	pH of HCl containing gluten.
gm.	N		
1	0.01	10	2.23
1	0.005	5	2.68
2	0.01		2.46
2	0.005	2.5	3.32
4	0.01		3.12
1	0.002	2.0	3.78
4	0.005	1.5	4.16
1	0.001	1.0	4.43
2	0.002		4.52
1	0.0005	0.5	4.93
2	0.001		4.98
4	0.002		5.00
2	0.0005	0.25	5.27
4	0.001		5.34
8	0.002		5.34
4	0.0005	0.125	5.43
8	0.001		5.55
8	0.0005	0.062	5.64
1	0	0	5.64
2			5.69
4			5.73
8			5.69

B

Gluten in 100 cc. solution.	Concentration of NaOH.	$R \cdot \left(\dfrac{NaOH}{gluten}\right)$	pH of NaOH containing gluten.
gm.	N		
8	0.00025	0.031	5.91
2	0.00012	0.062	5.97
4	0.00025		6.05
8	0.0005		6.06
1	0.00012	0.125	6.19
2	0.00025		6.39
4	0.0005		6.41
8	0.001		6.22
1	0.00025	0.25	6.87
2	0.0005		6.87
4	0.001		6.82
8	0.002		6.69
1	0.0005	0.5	7.72
2	0.001		7.53
4	0.002		7.45
8	0.004		7.15
1	0.001	1.0	9.63
2	0.002		9.37
4	0.004		9.28
1	0.002	2.0	10.08
2	0.004		10.70
4	0.01	2.5	10.76
1	0.004	4.0	11.26

II.

Further analysis of the system is made possible by measurements of the electrical conductivity of the aqueous phase.

In Table II, which takes account only of the acid systems, because of the greater consistency of the measurements, the concentration of free hydrochloric acid is calculated from the measurements of hydrogen ion concentration; that part of the conductivity due to hydrochloric acid is next calculated, and this is then subtracted from the observed con-

TABLE II.

Gluten in 100 cc. solution.	HCl original concentration.	pH	$\overset{+}{(H)}$	Combined HCl.	Conductivity.	Conductivity of free HCl.	Salt conductivity.	Corrected salt conductivity.	R	X
gm.	N		N							
1.0	0.01	2.23	0.0059	0.0041	2,851	2,450	401	353	86	150
	0.005	2.68	0.00209	0.00291	1,142	870	272	224	77	75
	0.002	2.78	0.000166	0.00184	255	69	186	138	75	45
	0.001	4.43	0.000037	0.000965	132	15.4	116.6	68	71	20
	0.0005	4.93	0.0000118	0.000490	89	4.9	84.1	36	(73)	10
	0.0000	5.64			48.5					
2.0	0.01	2.46	0.00347	0.00653	2,133	1,480	653	565	86	240
	0.005	3.32	0.00048	0.00452	605	199	406	318	70	90
	0.002	4.52	0.0000302	0.00197	234	12.6	221.4	133	67	35
	0.001	4.98	0.0000105	0.000991	132	4.35	127.6	39	(39)	
	0.0005	5.27	0.0000054	0.000497	137	2.24	134.8	46	(93)	
	0.0000	5.69			88.5					
4.0	0.01	3.12	0.00076	0.00924	1,113	316	797	649	70	200
	0.005	4.16	0.000069	0.00493	491	28.6	462.4	314	64	70
	0.002	5.00	0.000010	0.00199	276	4.15	271.8	123	62	25
	0.001	5.34	0.0000046	0.000997	270	1.91	268.1	120	(120)	
	0.0005	5.43	0.0000037	0.000498	219	1.54	217.5	69	(138)	
	0.0000	5.73			148.5					
8.0	0.002	5.34	0.0000046	0.001997	391	19.1	389.1	109	55	10
	0.001	5.55	0.0000028	0.000999	306	11.6	304.8	25	(25)	
	0.0005	5.64	0.0000023	0.0004997	278	09.6	277.0	−3		
	0.0000	5.69			280					

ductivity. The remainder, given in Table II as "salt conductivity," represents the effect of all other ions than those which may be hypothetically attributed to free hydrochloric acid. From this remainder the conductivity of the system in the absence of hydrochloric acid is subtracted and the result is called "corrected salt conductivity." It represents the effect of the action of the acid, chemically or other-

wise, to increase conductivity, aside from the direct effect of the ions arising from those molecules of acid which remain free in the solution. Finally this value is compared with the total amount of acid which has disappeared from the solution, and which may therefore be held responsible, directly or indirectly, for this action. This comparison is expressed in the form of a ratio designated in Table II as R.

It will be seen that there is a rough constancy of the ratio of corrected salt conductivity to combined acid. As might have been expected, this is not true in the lower ranges of acidity where the experimental errors, presumably of constant magnitude, are large in proportion to the effects of the acid added.

Disregarding the systems where the quantity of acid is small, it may also be seen that the numbers vary in a regular manner. The greater the hydrogen ion concentration, the greater are the values of R (Table III).

TABLE III.
Values of R.

pH	Gluten in 100 cc.			
	1 gm.	2 gm.	4 gm.	8 gm.
2.2	86	86	70	
2.6	77	70		
3.2	75		64	
3.8				
4.2	71	67	62	
4.5				55
5.0				
5.3				

In order to investigate this subject. we may provisionally assume that. however it comes about, the effect upon the conductivity of the formation of a given amount of protein chloride is constant. Of course this can be at best only a very rough approximation. As the acidity decreases. the values of R tend to approach a limit which we may take. for purposes of rough estimate, as 50, expressing the value in round numbers.

This makes possible an estimate. however hypothetical, of that increment of the conductivity which is due to protein chloride, or is

in any other way proportional to the quantity of combined acid. This value may then be subtracted from the corrected salt conductivity, thus giving as a final result that increment of the increase of conductivity which is not proportional to simple chemical action and which measures either the inaccuracies of the assumptions and approximations or the changes in the systems which are not due to phenomena already considered. The results of this calculation are given under X in Table II.

That part of the increase in conductivity which is proportional to protein salt formation may be supposed to result from the direct liberation of electrolytes by the union of the acid with the protein plus at least a moiety of the ionization of dissolved protein chloride.

The most obvious effects which have been neglected are the increasing rate of change of solubility of protein with increasing acidity and a corresponding change in the gluten mass, which involves the liberation of electrolytes. Both these factors ought to produce such a change in conductivity as is revealed by the data. For the protein which dissolves, whether chloride or not, will carry ions with it into the solution, and the disintegration of the gluten will favor the escape of electrolytes which might not otherwise find an open path into the aqueous phase.

It is therefore apparent that the simplest possible chemical phenomena, which are certainly all involved in the equilibria of the system, are quantitatively sufficient, within the range of accuracy of the measurements, to explain all the data. In short, the theoretical assumptions which are necessary upon purely chemical grounds are, within a very close approximation, also sufficient to account for the phenomena.

III.

In order to proceed a step further in the investigation, it is necessary to study the relation between electrolytes in the solution and in the gluten phase. The following experiment bears upon this point. A series of systems, consisting of different quantities of powdered gluten and 100 cc. of distilled water of specific conductivity 9.6, was allowed to stand at a temperature of 22°C., and from time to time the conductivity was measured. The results are included in Table IV.

TABLE IV.

Gluten in 100 cc. water.	Conductivity after						
	1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.	27 hrs.
gm.							
8			172.			192.	274.
4			137.			157.	178.
2	62.8	70.9	88.4	93.5	101.	104.	138.
1			58.9			63.4	72.8
0.5		35.7			42.6	40.7	
0.25	28.3	30.3	35.7	39.1	41.5	41.5	
0.125		27.4			31.6	28.3	

Making allowance for the conductivity of the water, and reducing the measurements to the effect per gm. of protein, this experiment yields the results of Table V, which indicate that the amount of elec-

TABLE V.

Gluten in 100 cc. water.	Relative solution of electrolytes per gm. of gluten after						
	1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.	27 hrs.
gm,							
8			20			23	33
4			32			37	42
2	27	31	39	42	46	47	64
1			49			54	63
0.5		52			66	62	
0 25	75	83	104	118	128	128	•
0.125		142			176	150	

trolytes contained in 1 gm. of the gluten preparation is approximately sufficient to give a specific conductivity at 25°C. of 200 to 100 cc. of water. This rough estimate accords satisfactorily with determinations of the ash of the gluten:

		per cent
I	....	0.59
II	....	0.61
Average	....	0.60

These experiments illustrate the importance of the relation between quantity of protein and volume of solution in such systems, even when gluten is added to distilled water. When the variation of solubility and hydration of proteins under the influence of electrolytes are also

TABLE VI.

Calculation of Conductivity of Ions Combined with 1 Gm. of Gluten at Different Hydrogen Ion Concentrations.

$R\left(\frac{HCl}{gluten}\right)$	Weight of gluten.			
	1 gm.	2 gm.	4 gm.	8 gm.
10	1,465			
5	1,123	1,191		
2.5		930	951	
2.0	775			
1.5			594	
1.0	483	498		
0.5	346	341	339	
0.25		235	236	255
0.125			197	214
0.0625				166
0	155.5	157.7	164.8	165.5
$R\left(\frac{NaOH}{gluten}\right)$				
0.031				172
0.062			171	188
0.125		180	179	186
0.25	181	194	198	203
0.5	225	227	231	239
1.0	277	296	308	
2.0		413		
2.5			591	
4.0	509			

taken into account, it may be clearly seen how illusory are all conclusions which leave such factors out of consideration. At the same time, there is every indication in the regularity of the data that the final condition of equilibrium is a simple one. But this condition is slowly attained, and by a somewhat devious path.

The immediate conclusion to be drawn from these measurements is that the electrolytes originally present in the gluten are sufficient,

per gm. of gluten, to give a conductivity of approximately 200 to 100 cc. of water. This may now be used in further study of the data upon conductivity. For this quantity, plus the electrical conductivity of the solution of acid or alkali added to the gluten, minus the electrical conductivity of the resulting solution, may be taken as a roughly approximate measure of the quantity of electrolyte in union with protein. It is, however, a measure which cannot be directly employed for comparison of the acid systems with the alkaline systems, because of the considerable difference in conductivity of hydrochloric acid and sodium hydroxide. And of course the various salts of the original gluten are also not equal in conductivity to the acid, to the alkali, or to each other.

The results of such calculations of the conductivity of electrolytes in union with gluten are collected in Table VI. It may easily be seen that the data vary in a regular and intelligible manner.

IV.

In the light of the above facts the swelling of gluten in solutions of acid and alkalies may now be considered. The data of Table VII include observations upon the weight of the wet, swollen, coherent mass, upon the weight of dry gluten contained therein, as well as calculations of the amount of gluten dissolved and of the ratio of wet gluten to dry material contained within it (the true swelling).

Quantitatively the results are not at all points satisfactory. The following conclusions are possible. First, the weight of swollen gluten bears no relation to the true swelling (hydration) because of the marked variations in the quantity of protein dissolved. Second, it bears no direct relation to the hydrogen ion concentration, except when quantities of gluten and solution are kept constant. Third, the chief factor in determining the weight of swollen gluten is the quantity of protein which has been dissolved away from it. Fourth, the amount of gluten dissolved is greater the greater the acidity. Fifth, the true swelling of gluten is greater the greater the acidity. There also seems to be a tendency for the true swelling of gluten to increase as the relative quantity of gluten increases. This corresponds to a relatively greater amount of electrolyte present in the swollen mass.

TABLE VII.

Measurements on Gluten (5.6 Gm.) in Equilibrium with Solutions of HCl and NaOH at Different Reactions.

pH	R	Gluten per 100 cc. solution.			
		1 gm.	2 gm.	4 gm.	8 gm.
	$\left(\dfrac{HCl}{gluten}\right)$	Weight of swollen gluten (A).			
		gm.	*gm.*	*gm.*	*gm.*
5.3	0.25		11.5	11.1	13.1
5.5	0.125			12.9	14.3
5.6	0.0625				14.5
5.7	0.0000	12.9	13.7	15.3	15.7
	$\left(\dfrac{NaOH}{gluten}\right)$				
5.9	0.0312				15.8
6.0	0.0625		14.2	15.4	16.0
6.3	0.125	14.2	15.1	15.7	15.6
6.8	0.25	15.1	15.1	15.2	15.4
7.5	0.50	14.6	14.8	15.0	15.3
9.4	1.00	13.6	13.9	14.3	
	$\left(\dfrac{HCl}{gluten}\right)$	Weight of dry gluten in 12 gm. of swollen gluten (B).			
5.3	0.25		3.75	3.74	3.22
5.5	0.125			3.90	3.89
5.7	0.0000				4.08
	$\left(\dfrac{NaOH}{gluten}\right)$				
5.9	0.0312				3.79
6.0	0.0625		3.93	3.93	3.89
6.3	0.0125	4.04	3.93	3.93	4.01
6.8	0.25	3.99	4.05	4.08	4.00
7.5	0.50	4.16	4.18	4.05	
9.4	1.00		4.12		
	$\left(\dfrac{HCl}{gluten}\right)$	Amount of gluten dissolved $\left(5.6-\dfrac{A\cdot B}{12}\right)$.			
5.3	0.25		2.01	2.14	2.08
5.5	0.125			1.41	0.96
5.7	0.000				0.67
	$\left(\dfrac{NaOH}{gluten}\right)$				
5.9	0.0312				0.62
6.0	0.0625		0.95	0.56	0.41
6.3	0.125	0.82	0.65	0.46	0.39
6.8	0.25	0.58	0.50	0.43	0.47
7.5	0.50	0.54	0.44	0.54	
9.4	1.00		0.83		

TABLE VII—*Concluded*.

pH	R	Gluten per 100 cc. solution.			
		1 gm.	2 gm.	4 gm.	8 gm.
	$\left(\dfrac{HCl}{gluten}\right)$	Swelling of gluten $\left(\dfrac{12}{B}\right)$.			
		gm.	gm.	gm.	gm.
5.3	0.25		3.20	3.20	3.72
5.5	0.125			3.08	3.08
5.7	0.000				2.94
	$\left(\dfrac{NaOH}{gluten}\right)$				
5.9	0.0312				3.17
6.0	0.0625		3.05	3.05	3.08
6.3	0.125	2.97	3.05	3.05	2.99
6.8	0.25	3.01	2.96	2.94	3.00
7.5	0.50	2.88	2.87	2.96	
9.4	1.00		2.92		

V.

Finally, we have measured the viscosity of swollen gluten with the help of the viscosimeter previously described. In every case 12 gm. of the swollen gluten were introduced into the apparatus.[3] The results of these measurements are included in Table VIII.

It should be understood that these measurements are of very moderate accuracy which, nevertheless, is sufficient for the present purpose. Evidently there is a well marked minimum of viscosity near a hydrogen ion concentration corresponding to pH 5.7. This minimum, as will be shown in a later paper, while due chiefly to the hydrogen ion concentration, is in a secondary manner influenced by the amount of electrolyte present in the system, and varies with this factor as well as with the acidity. The electrolyte concentration determines the great variation in viscosity measurements in the systems where the protein is suspended in distilled water. It will be shown in a later paper that the value of 3.0 for the system containing 8 gm. of gluten is most nearly comparable with the other data of the table.

[2] Henderson, L. J., Fenn, W. O., and Cohn, E. J., *J. Gen. Physiol.*, 1918–19, i, 387.

The measurements on smaller quantities of gluten in distilled water are accordingly disregarded. Averaging all the other values of Table VIII, which appear to be fairly comparable, the last column is obtained. This column represents more truly than the individual data the effect of variation of hydrogen ion concentration upon viscosity. It is graphically represented in Fig. 1.

The marked minimum in the viscosity of the gluten seems not to be dependent upon a single property. For it does not coincide with

TABLE VIII.

Viscosity Measurements.   Time in Minutes.

pH	R	Gluten per 100 cc. solution.				
		1 gm.	2 gm.	4 gm.	8 gm.	Average.
	$\left(\dfrac{HCl}{gluten}\right)$	Weight, 350 gm.				
5.3	0.250				16.0	16.0
5.5	0.125			10.8	7.5	9.2
5.6	0.062				5.8	5.8
5.7	0.000	55.0	28.0	5.5	3.0	3.0
	$\left(\dfrac{NaOH}{gluten}\right)$	Weight, 450 gm.				
5.9	0.0312				2.8	2.8
6.0	0.0625		5.8	5.0		5.4
6.3	0.125	6.3	6.8	4.0	5.8	5.7
6.8	0.25	6.3	5.0	7.0	6.3	6.2
7.5	0.50	12.5	9.8	10.3	15.3	12.0
9.4	1.0	18.0	17.5	13.5		16.3

the isoelectric point—according to Wood and Hardy[4] probably between 10^{-7} N and 10^{-8} N—and it does not correspond with the true swelling, or hydration. It is, moreover, as the observations in the presence of distilled water show, greatly influenced by the concentration of electrolytes. This last point will be fully established in a later paper. Finally, it appears to be largely variable with time, so that a process of "setting" may be suspected.

[4] Wood, T. B., and Hardy, W. B., *Proc. Roy. Soc. London, Series B*, 1909, lxxxi, 39.

In short, the viscosity of gluten seems to depend upon the hydrogen ion concentration, upon the amount of water and of electrolytes present in the swollen mass, and at times upon the age of the system. These are, however, the factors whose importance was to have been expected. A more complete interpretation of their mode of action is at present impossible. But the well marked minimum of viscosity in an acid range of reaction is plain, and, as we hope to show, of decisive practical importance in bread making.
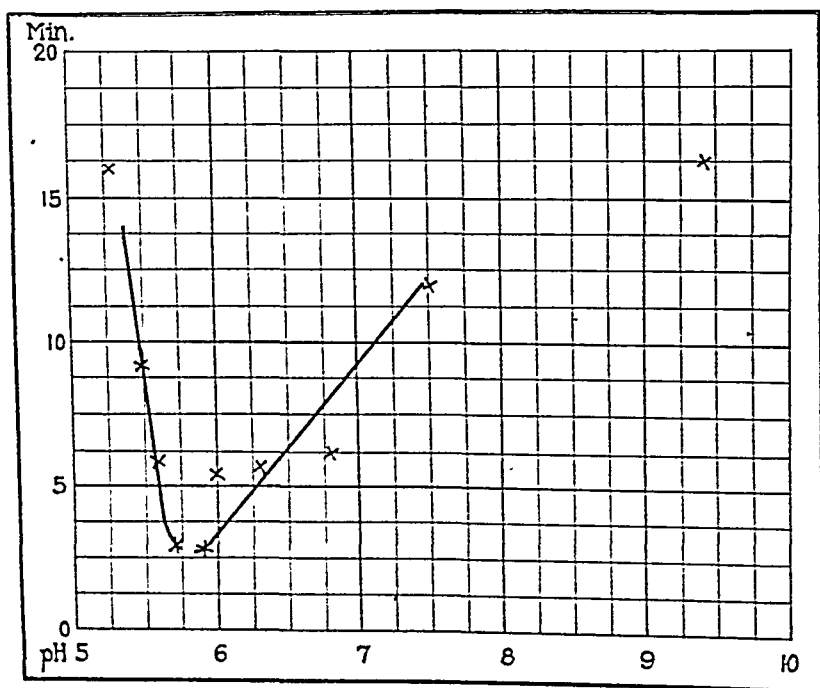
FIG. 1.

SUMMARY.

In this paper there are reported studies of the acid-base equilibrium in systems containing gluten suspended in solution of hydrochloric acid and sodium hydroxide. The studies have involved measurements of the hydrogen ion concentration, of the electrical conductivity, and of the solution of the proteins. Further, measurements have

been made of the swelling and of the viscosity of the gluten component of such systems.

The results seem to show that simple chemical phenomena are most important in such systems, and that the modifications of these, resulting from colloidal and heterogeneous characteristics, are of secondary importance in determining the condition of equilibrium, though somewhat more significant in the progress of the system toward the condition of equilibrium.

RELATION BETWEEN THYROID GLAND, METAMORPHOSIS, AND GROWTH.

By EDUARD UHLENHUTH.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

It has been demonstrated by a number of workers that the active principle of the thyroid gland in the causation of amphibian metamorphosis is iodine. Recently Swingle[1] has obtained definite proof that inorganic iodine when fed to larvæ of *Salientia* induces metamorphosis in a short time after the beginning of the feeding. On the other hand, it has been shown by the writer[2] that the retarding influence of the feeding of thymus upon amphibian metamorphosis is due to the absence from the thymus gland of a substance required for metamorphosis, and it is possible that this lacking substance is iodine. Although minute amounts of iodothyrin have been found in some thymus glands, it is likely that the amounts found are insufficient to produce metamorphosis and that some thymus glands may not contain the iodine at all. This would account for the variability of the results obtained with thymus feeding.

Swingle[1] also subjected tadpoles deprived of their thyroid to an iodine diet; although such larvæ when kept on a normal diet never metamorphose, they very soon metamorphosed when fed on iodine crystals.

Hence it is manifest that iodine is one of the substances involved in the causation of amphibian metamorphosis. The quantities contained in normal food are, however, so small as to have no immediate effect upon the organism. If the larvæ have no thyroid glands, the small quantities of iodine taken up with the food apparently cannot be retained by the organism and the iodine leaves the body without

[1] Swingle, W. W., *J. Exp. Zool.*, 1919, xxvii, 397.
[2] Uhlenhuth, E., *J. Gen. Physiol.*, 1918–19, i, 305.

bringing about metamorphosis. If, however, a thyroid gland is present, all or most iodine taken up by the organism is retained and stored up in the thyroid. At a certain time during the life of the larva the thyroid suddenly begins to excrete the iodine stored up during the larval period, and metamorphosis results.

From this it is evident that under normal conditions the iodine is not the only substance needed in metamorphosis; there must be still another substance which, when present in a certain quantity, causes the thyroid to excrete the thyroid hormone. The experiments to be reported in this article not only furnish evidence of the actual existence of such an excretor substance, but they also demonstrate that it is evolved during the processes which lead to the growth of the organism.

Metamorphosis and Rate of Growth in Worm-Fed Larvæ.

In eleven series of larvæ of the species *Ambystoma opacum* the rate of growth during the larval period of active growth has been determined in the following way.

In each series the time of metamorphosis was recorded for each single individual; this can be done very accurately in this species if the first shedding of the skin and the reduction of the gills to mere stumps without fringes are taken as the indication of metamorphosis, both phenomena occurring within a day. That these two processes are actually controlled by the influence of the thyroid, in contradistinction to many other processes, has been indicated already in former articles[3] and will be shown in detail in a later publication. From the values obtained in this way the average length of time of the larval period was calculated for each series (Table I).

Each individual was measured once a week and the average sizes obtained from those values for each series were plotted in curves. At the time of metamorphosis a sharp drop of the curve takes place, due to a discontinuation of growth, and even a diminution of the size of the animals which may last for one or several weeks before growth is resumed. In Series A 1916, the curve of which is shown in Fig. 1,

[3] Uhlenhuth, E., *J. Exp. Zool.*, 1917–18, xxiv, 237; 1918, xxv, 135; *J. Gen. Physiol.*, 1918–19, i, 305.

this drop occurred in the 24th week, though less distinctly than in most of the other series.   From the average sizes reached in each series when the drop occurred and from the time when the drop took place, the rate of growth was calculated in day-millimeters, as indicated in Column 3 of Table I.

In comparing the length of the larval period for each series with the corresponding rate of growth it becomes evident that the smaller the rate of growth observed, the later metamorphosis took place. Furthermore, the rates of growth appear to be proportional to the

TABLE I.

Rate of Growth (R) in Worm-Fed Ambystoma opacum during Larval Period of Active Growth (Calculated from Averages).

Series.	Age at metamorphosis.	R	R × A
	days		
C 1916..	245	0.27	66
A 1916..	182	0,31	56
E 1917..	161	0.36	58
D 1917..	127	0.48	61·
W$_K$ 1917	106	0.58	62
W$_{Na}$ 1917	100	0.58	58
W$_{Mg}$ 1917	100	0.58	58
W 1917..	97	0.62	60
W$_{Ca}$ 1917....................................	96	0.62	60
C 1917..	80	0.79	63
XIV 1918..	70	0.83	58
Average..			60

velocity of metamorphosis; for the product of the rate of growth into the duration of the larval period (R × A) is constant, as may be seen from Table I, the average value of R × A being 60.

This means that during the process of growth a substance is evolved which when present in a definite amount induces metamorphosis, provided that the larvæ have been fed on normal food which apparently contains enough iodine to furnish the other substance (iodine) required for metamorphosis in a sufficient quantity; the greater the rate of growth the quicker that quantity of the first substance is formed which is required to induce the secretory action of

the thyroid gland. Hence besides iodine still another substance is needed in the amphibian metamorphosis; namely, the excretor substance which causes the thyroid to excrete the stored up iodine.

It may be mentioned here that the sudden drop of the growth curve at the time of metamorphosis may be explained if we assume that the thyroid, when it is stimulated by the excretor substance, excretes at
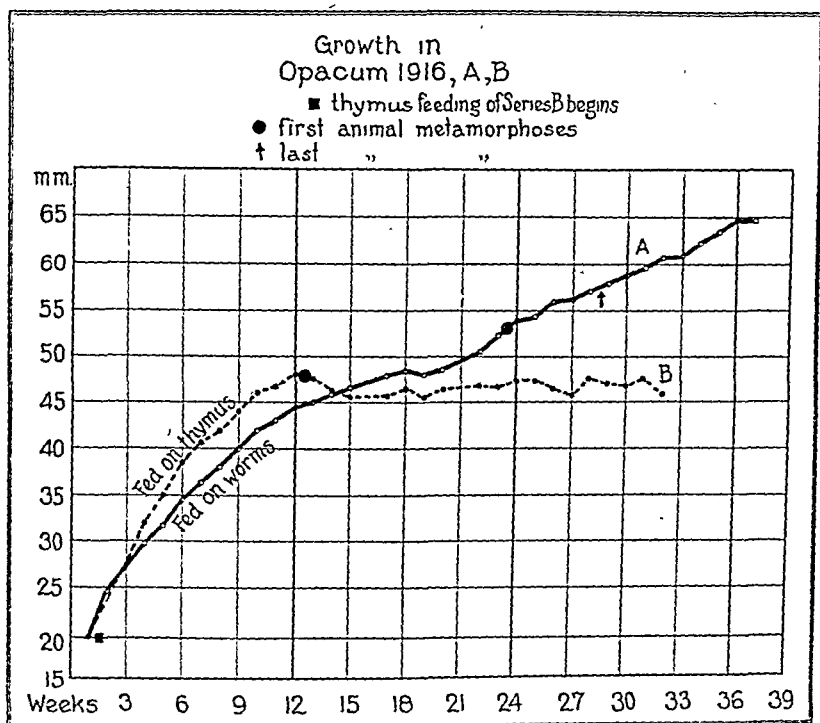

FIG. 1.

first an overdose of iodine, while later the excretion becomes less energetic. Janney[4] has shown that while an overdose of thyroid hormone leads to a negative nitrogen balance and a loss in weight and size, a certain minimum dose results in a positive nitrogen balance and a gain in weight and size.

[4] Janney, N. W., *Arch. Int. Med.*, 1918, xxii, 187.

Metamorphosis and Rate of Growth in Thymus-Fed Larvæ.

In a recent publication Janney[1] has shown that while a certain minimum amount of the thyroid hormone results in an increased protein breakdown, it finally leads to a positive nitrogen balance and an increase in weight and size, since it not only accelerates the protein breakdown but at the same time facilitates the assimilation of the nitrogen into the proteins of the body tissues. This is shown also in the metamorphosis of the amphibians where the initial breakdown of the tissues, as demonstrated, for instance, by Morse[5] on tadpoles, and the decrease in size, resulting probably, as suggested above, from the excretion of an overdose in the beginning of the functional period of the thyroid, are followed soon by an increase in size and weight.

On the other hand, Janney's experiments have shown that in certain diseases, such as exophthalmic goiter, the normal synthesis by the thyroid of the thyroid hormone from iodine and certain organic substances is disturbed, and on the basis of Swingle's experiments we may assume that in these diseases the thyroid is unable to retain the iodine consumed by the organism with the food. This assumption is supported by the fact, as mentioned by Janney, that in exophthalmic goiter the thyroid frequently is found very poor in iodine. But since in this case the excretory function of the thyroid remains undisturbed, the thyroid is excreting, instead of the hormone, certain substances, probably the indole-containing amino-acid tryptophane which normally is used to build up the hormone, but which in itself is toxic causing a permanent protein breakdown without facilitating assimilation of the food nitrogen; consequently a negative nitrogen balance is brought about and a permanent loss of weight.

If we apply this hypothesis to amphibian metamorphosis, we should expect that in such larvæ, which have not been able to take up an amount of iodine sufficient for metamorphosis and whose thyroid consequently was unable to develop the normal hormone, the action of the excretor substance would lead to a prolonged and increased loss in size of the larvæ, without resulting metamorphosis, since in these animals excretion by the thyroid would commence as soon as the excretor substance reached the amount required, but the excreted

[5] Morse, W., *Biol. Bull.*, 1918, xxxiv, 149.

substance would be the toxic substance tryptophane instead of the normal hormone.

This expectation is actually fulfilled in the thymus-fed larvæ of *Ambystoma opacum*, as is shown by Curve B, Fig. 1. In this curve is represented the growth of a series of larvæ of the same age and from the same mother as the larvæ used for the experiment plotted in Curve A; both series were kept under the same conditions, but while Series A was fed on earthworms, Series B was fed on thymus. The thymus-fed larvæ grew normally in the beginning—and in this particular series even more quickly than the controls, a fact explained by the writer in a previous paper.[6] At the 13th week we observe a sharp drop in Curve B and hereafter growth was stopped and never resumed again. Exactly the same results were obtained for the thymus-fed larvæ of *Ambystoma maculatum* and *Ambystoma tigrinum*.

The time when the drop of the curve takes place in the thymus-fed animals is that at which metamorphosis should have occurred if they had received normal food. This was not only suggested by the general appearance of these larvæ and by the fact that a few larvæ actually did metamorphose (in Series B·1916 only one larva metamorphosed at this time), but it can be proved if we calculate the time of metamorphosis from the value of the product R × A for the worm-fed larvæ. This has been done in Table II for eight thymus-fed series of *Ambystoma opacum*. Again the product R × A was calculated from the duration of the larval period and the rate of growth, the latter value being obtained as in the worm-fed larvæ from the growth curve up to the point where the drop occurred. First we notice that the product R × A (Table II) in the first three series is far above the average value of R × A as obtained for the worm-fed series. This is due to the fact that the larvæ of these series did not metamorphose at a time proportional to their rate of growth but much later, due to the absence of iodine at this time. The time at which they should have metamorphosed if iodine had been present in the food in a normal amount, can be calculated, however, from the product R × A in the worm-fed series, which is 60, and the rate of

[6] Uhlenhuth, E., *Proc. Soc. Exp. Biol. and Med.*, 1917–18, xv, 37.

growth in each particular thymus-fed series. We find, then, that in the first three series metamorphosis was greatly retarded (Column 5, Table II). In Series D 1916 metamorphosis should have taken place at 162 days instead of at 207 days, in Series B 1916 at 109 days instead of at 133 days, and in Series T_{Ca} 1917 at 113 days instead of at 122 days. In the other series the retardation of metamorphosis, if there was any at all, was only slight; how far this was due to the special treatment these thymus-fed animals received will not be discussed here. It may be mentioned, however, that in the last series (T 1917) the retardation of metamorphosis was prevented probably

TABLE II.

Rate of Growth (R) in Thymus-Fed Ambystoma opacum during Larval Period of Active Growth (Calculated from Averages).

Series.	Age at metamorphosis.	R	$R \times A$	Age at which animal should metamorphose.
	days			*days*
D 1916......................	207	0.37	77	162
B 1916......................	133	0.55	73	109
T_{Ca} 1917....................	122	0.53	65	113
B 1917......................	122	0.50	61	120
T_{Na} 1917	122	0.50	61	120
T_{Mg} 1917.....................	109	0.58	63	103
T_K 1917	104	0.57	59	105
T 1917......................	101	0.60	61	100
Average..			65	

by adding parathyroid to the thymus diet; since the parathyroids are known to contain small amounts of iodine, it is probable that the thyroid of these larvæ was apparently able to store up enough iodine to permit normal thyroid excretion and metamorphosis when the excretor substance began to act.

The number of days at which the second thymus-fed series (B 1916) should have metamorphosed, *i.e.* at which the excretor substance began to act, is 109. This approaches very closely the time when the drop of the curve occurred (95 days), if we consider that the value 109 was calculated only from an average value of $R \times A$.

If prevention of metamorphosis by the absence of iodine, but in the presence of a thyroid gland, is accompanied by a check in growth (for the reasons given above), we should expect that prevention of metamorphosis in the absence of a thyroid gland would not be accompanied by an abnormal check of growth. Hence amphibian larvæ deprived of their thyroids should be able to complete the normal growth of the species without any disturbances. That this is actually the case is shown in Allen's tadpoles which were deprived artificially of their thyroid glands and which reached frequently enormous sizes, becoming real giant larvæ. A similar phenomenon is found in such forms as *Typhlomolge rathbuni*, which for some reason do not develop a thyroid gland—as discovered by Emerson[7]—and hence remain permanently in a larval condition, but which seem able to grow for years without disturbances.

Thus the experiments reported in this article seem to prove that metamorphosis of the normal larvæ of *Ambystoma opacum* which possess a thyroid depends not only upon the presence of a sufficient amount of iodine in this gland but also on the presence of the action of a second substance inducing the excretion of the iodine by the thyroid gland. By means of this hypothesis we are able to explain why thymus-fed larvæ suddenly stop growing at the time when metamorphosis should occur, without, however, metamorphosing, and why species not possessing thyroid glands, such as the *Typhlomolge*, can complete their growth without disturbance.

Metamorphosis and Temperature.

There is still another phenomenon which lends itself readily to explanation on the basis of the assumption of an excretor substance. It is a well known fact that growth is retarded at low temperature, and since we have seen that the excretor substance is evolved during growth, it is not surprising that metamorphosis also should be retarded at low temperature. This has long been observed by many students of amphibian metamorphosis. But what remains unexplained is the fact that amphibian larvæ when kept in low temperature are always much larger at the time of metamorphosis than

[7] Emerson, E. T., *Proc. Boston Soc. Nat. Hist.*, 1905, xxxii, 43.

they would be if kept at high temperature. We have observed this phenomenon frequently and in looking for an explanation have tried to find a relation between the size of the larvæ and the age at the time of metamorphosis, similar to that existing between the latter quantity and the rate of growth. Such a relation, however, does not exist.

But if we assume the action of an excretor substance in metamorphosis, the phenomenon in question can be readily explained. Comparing $R \times A$ for the first worm-fed series (C 1916) in Table I, which was kept at a temperature of 10°C. below that of the other series, with the rest of the series of Table I, we observe that it is very high (10 per cent) above the average, which would indicate that metamorphosis in this series was more retarded than the corresponding rate of growth would demand. Since in this series a drop of the growth curve similar to the drop of the growth curve of the thymus-fed animals did not occur, this case of undue retardation of metamorphosis cannot be explained in the same way as in the thymus-fed larvæ, *i.e.* by asuming that from a lack of iodine in the thyroid gland a destructive compound was excreted by the thyroid. The only way to explain this case of retardation is to assume that at low temperature less excretor substance is evolved than at high temperature during an equal rate of growth processes. That this should be possible is not at all surprising, but was to be expected since it is well known that the temperature coefficients for different physiological processes may differ greatly. Loeb, for instance, pointed out that not only the temperature coefficients but also their variations at the lower and upper temperature scale differ considerably in different physiological processes.[5]

But if the amount of excretor substance produced by an equal rate of growth is less at low temperature than at high temperature, the animals kept at low temperature must grow longer at an equal rate than those kept at high temperature before that amount of excretor substance is produced which is required to bring about thyroid excretion. Consequently the low temperature larvæ must reach a larger size than the high temperature larvæ, before they can metamorphose.

[5] Loeb, J., Mechanistic conception of life, Chicago, 1912, 212.

The same phenomenon was observed also in the thymus-fed larvæ (Series D 1916, Table II) and since here the lack of iodine and that of the excretor substance are combined, it is not surprising that the value for R $\times$ A is still higher (28 per cent) above the average than in the low temperature series of the worm-fed larvæ (Series C 1916, Table I).

Thus with the hypothesis of an excretor substance we can explain a phenomenon which for a long time was confusing to experimental biologists as well as to systematists.

<center>SUMMARY.</center>

1. Two substances are involved in amphibian metamorphosis as studied in *Ambystoma opacum:* first, iodine, which is taken up by the food, and second, an excretor substance, which is evolved during the processes of growth and serves to induce the excretory function of the thyroid gland.

2. This explains why in larvæ, whose metamorphosis is inhibited by lack of iodine, growth is checked at the time when metamorphosis should occur; for at this time the excretor substance commences to act and this results, if iodine is absent, in the excretion by the thyroid of toxic substances which cause the breakdown of proteins and consequently a decrease in size of the larvæ.

3. Larvæ whose metamorphosis is inhibited by extirpation of the thyroid or by the hereditary lack of a thyroid (as is the case in *Typhlomolge*) can grow normally, since in them the action of the excretor substance cannot result in the excretion by the thyroid of a toxic growth-inhibiting substance.

4. At low temperature less excretor substance is produced than at high temperature during an equal rate of growth; therefore larvæ kept at low temperature reach a larger size than larvæ kept at high temperature, before they metamorphose.

AMPHOTERIC COLLOIDS.

IV. The Influence of the Valency of Cations upon the Physical Properties of Gelatin.

BY JACQUES LOEB.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

I. INTRODUCTION.

In 1901 and 1902 the writer[1] published a series of investigations in which he showed that low concentrations of bivalent cations, practically without regard to their chemical nature, *e.g.* Mg, Ca, Sr, Ba, Zn, Co, Pb, etc., inhibited the toxic action of high concentrations of salts with univalent cations, upon the eggs of *Fundulus*. Trivalent cations like $AlCl_3$ and $CrCl_3$ seemed to act still more effectively than the bivalent ions though it was obvious that secondary influences (*c.g.* the high hydrogen ion concentration) restricted the limit of their antagonistic influence. This influence of valency the writer attributed to the effect of the electric charges of the ions upon the physical state of the colloidal material. He also found that polyvalent anions, *e.g.* SO_4, oxalate, citrate, etc., had under the same conditions no antagonistic effect. Since that time cases of an antagonistic action of polyvalent anions have come to light, but this phenomenon is not only less common but also less striking than the antagonistic effect of polyvalent cations.

It had been shown in the writer's preceding publications on gelatin[2] that the salts of gelatin with univalent cations possess a comparatively high osmotic pressure, a high viscosity, a high degree of swell-

[1] Loeb, J., *Arch. ges. Physiol.*, 1901–02, lxxxviii, 68; *Am. J. Physiol.*, 1901–02, vi, 411.

[2] Loeb, J., *J. Biol. Chem.*, 1918, xxxiii, 531; xxxiv, 77, 395, 489; xxxv, 497; *J. Gen. Physiol.*, 1918–19, i, 39, 237, 363.

ing, and a high alcohol number, while the gelatin salts with bivalent cations have a much lower osmotic pressure, lower viscosity, etc. It was also found that the addition of a certain amount of a salt with a bivalent or polyvalent cation depresses the effect of salts with monovalent cation on osmotic pressure, viscosity, swelling, etc., of the gelatin.

Pauli[3] and Michaelis[4] seem to ascribe the variations in the swelling and the viscosity of protein solutions to variations in the degree of ionization and to a "hydratation" they assume to be connected with the ionization of the protein. According to this view we should have to assume that sodium gelatinate has a higher osmotic pressure than calcium gelatinate of the same concentration, because the former is more strongly ionized. In an earlier paper the writer tentatively accepted Pauli's hypothesis, but a closer scrutiny of the literature showed that neither Pauli nor Michaelis measured the effect of electrolytes upon the conductivity of their protein solutions, probably on account of the fact that they did not remove the excess of electrolyte after it had acted on the protein. The writer's method of removing the excess of electrolytes after they have had time to react with the gelatin made measurements of conductivity possible, and these measurements in connection with measurements of osmotic pressure and of the quantity of metal in combination with the gelatin led to a very definite explanation of the influence of the valency of ions on the properties of gelatin. *With the same equivalent of metal in combination with a given mass of gelatin the maximal osmotic pressure of a 1 per cent solution of gelatin salts with univalent cation, e.g. Na gelatinate, is almost exactly three times as great as that of gelatin salts with a bivalent metal, e.g. Ca gelatinate, while the conductivities of the solutions of the two types of gelatin differ little or not at all.* This indicates that the gelatin salts with univalent metal have at the point of maximal osmotic pressure about three times as many particles in solution as the same mass of gelatin salts with bivalent metal, while the number of electrical charges is about the same in both cases. The identity of the conductivities of gelatin salts of the type of sodium gelatinate and calcium

[3] Pauli, W., *Fortschr. naturwiss. Forschung*, 1912, iv, 245.

[4] Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914.

gelatinate proves that the difference in the influence of the valency of the cation upon the physical properties of gelatin cannot be ascribed to a difference in the degree of ionization of the two types of salts, but is due to some other as yet unknown factor. It seems possible to explain all the phenomena on the basis of the tentative assumption that gelatin salts with a bivalent cation dissociate into cations and aggregates of four, or six, or eight gelatin ions, each individual gelatin ion of the aggregate retaining its original negative charge, while the anions of salts of sodium gelatinate consist of only one gelatin ion each. The quantitative data suggest that the number of gelatin anions contained in each aggregate is of a stoichiometrical order, being a simple multiple of the number representing the valency of the polyvalent metal ion.

II. *Amount of Alkali Combining with Gelatin.*

We used powdered Cooper's non-bleached gelatin which is impure, having a pH of about 7.0 and consisting to a large extent of calcium gelatinate. It is necessary to purify this gelatin before using it by bringing it to the isoelectric point pH = 4.7.[5] This is done by putting 1 gm. of gelatin for $\frac{1}{2}$ hour into 100 cc. of 3 M/1024 HCl or M/128 acetic acid, then putting the gelatin on a filter, allowing the excess of solution to drain off, and washing the gelatin two or three times with 25 cc. of distilled water at 5°C. If we wish to transform the pure isoelectric gelatin into a metal gelatinate we treat it subsequently with the hydroxide of the metal—*e.g.* NaOH, Ca(OH)₂, etc.—with which we wish the gelatin to combine.

We always used finely powdered gelatin rendered isoelectric in the manner described. When we intended to prepare sodium gelatinate we treated different doses of isoelectric gelatin of 1 gm. each with 75 or 100 cc. of a NaOH solution varying from M/4 to M/8192 NaOH, after-

[5] Isoelectric gelatin does not react with neutral salts like NaCl but will react with NaOH, since in this latter case the pH is raised beyond that of the isoelectric point. Common Cooper's gelatin, with a pH = 7.0, consisting of gelatin salts, especially Ca gelatinate, will be influenced in the same way by a treatment with NaOH as with NaCl, since in both cases a replacement of Ca by Na will occur. This is supported by the writer's previously published papers.

wards washing away the excess of alkali. In each case sodium gelatin-
ate was formed but the amount formed varied with the concentration
of the alkali solution used; and the pH varied correspondingly. It
was of importance to measure the amount of Na or Ca in combina-
tion with the gelatin, to make sure that we were dealing with phenom-
ena of a stoichiometrical character; it was especially necessary to
make sure whether or not Na and Ca combine with gelatin in equiva-
lent proportions. The metal gelatinate used for this purpose was not
only washed as indicated, but was also dialyzed over night through
collodion bags against 400 cc. of distilled water.

Our former experiments[6] allow us to measure the amount of metal
contained in a given mass of gelatin when the pH is known. Metal
gelatinates can only exist on the alkaline side from the isoelectric
point of gelatin, this point being defined by a pH = 4.7. The Na or K
or Ca in combination with the gelatin at each pH can be calculated in
the following way. We determine the cc. of 0.01 N NaOH required to
bring 25 cc. of 1 per cent isoelectric gelatin of different pH (lying be-
tween 4.7 and 7.0) to the point of neutrality (pH = 7.0). By deduct-
ing this value from the quantity required to bring 25 cc. of 1 per cent
gelatin solution from the isoelectric point to pH = 7.0—which was al-
ways found to be about 4.5 cc. of 0.01 N NaOH[7]—we obtain the amount
of 0.01 N Na or of $\frac{1}{2}$ Ba or $\frac{1}{2}$ Ca in combination with the 25 cc. of 1
per cent gelatin at any pH between 4.7 and 7.0. When pH is >
7.0 we ascertain the amount of 0.01 N HCl required to bring the 25
cc. of gelatin to pH = 7.0, and add this to the value 4.5. In Fig. 1 the
abscissæ are the pH, the ordinates the cc. of 0.01 N Na, K, or $\frac{1}{2}$ Ba
found in combination with 25 cc. of gelatin for each pH. The results
of three different experiments with the three different alkalies named
are plotted (Fig. 1), showing the degree of agreement of the results.
The curves are exactly the same whether gelatin has been treated
with KOH, NaOH, LiOH, Ba(OH)$_2$, or Ca(OH)$_2$. It is also obvious
from the curves that we are dealing with a simple salt formation of

[6] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 363.

[7] In our preceding paper we determined the cc. of 0.01 N NaOH required to
bring 25 cc. of 1 per cent gelatin from the isoelectric point to the turning point of
phenolphthalein (pH = 9.0) and found 5.5, which agrees with our present result
where we neutralize to pH = 7.0 instead of to 9.0.

a stoichiometrical nature and that one Ba or Ca replaces two Na (or two Li, K, or NH₄). The maximum amount of salt formed is practically reached not far beyond pH = 8.0, which is so near the point of neutrality that practically no corrections for the values for titration and only slight corrections for the values for conductivity are required. Our conductivity curves are the corrected curves, *i.e.* from the measured values the conductivities of the pure alkali solutions of the same pH are deducted.
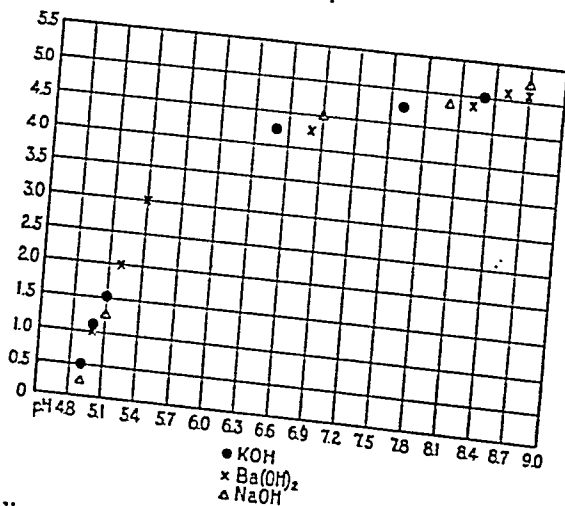

FIG. 1. Ordinates represent amount of Na, K, and ½ Ba (expressed in cc. of 0.01 N Na) in combination with 25 cc. of 1 per cent gelatin solution, previously treated with NaOH, KOH, or Ba(OH)₂ respectively and freed from the excess of salt by washing. Abscissæ represent pH of the solution. Curves in all three cases are identical, showing that one Ba replaces two Na or K.

III. Action of Different Alkalies on the Conductivity and Osmotic Pressure of Gelatin.

In order to obtain constant results we brought the powdered gelatin, as stated, to the isoelectric point by treating it for 30 minutes with M/128 acetic acid at 20°C. The gelatin was then put on a filter, the acid allowed to drain off, and then it was washed twice with 25 cc. of distilled water at about 5°C. Then such gelatin, while on the filter, was perfused three times with 25 cc. of a solution of NaOH or KOH,

etc., of a definite concentration, varying from $M/4$ to $M/8192$ at 15°C. During this perfusion the powdered gelatin was sufficiently stirred to allow intimate contact between all the gelatin particles and the alkali, and then after all the excess of alkali had been allowed to drain off the mass on the filter was washed once with 25 cc. of H_2O at 5° and
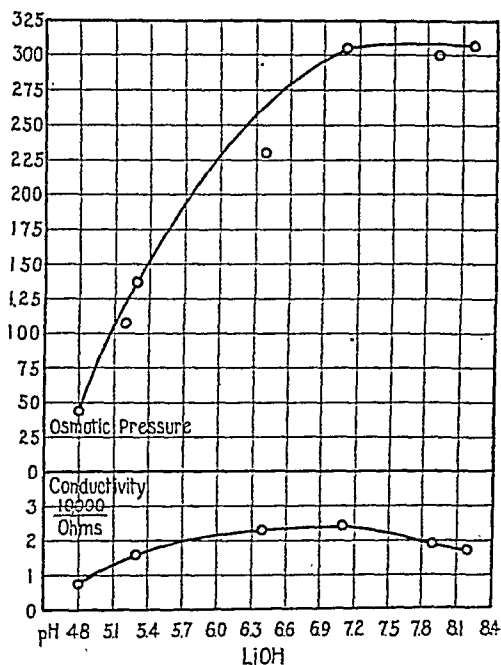
FIGS. 2 to 7. Curves for osmotic pressure (in terms of mm. of a 1 per cent gelatin solution) and conductivity $\left(\dfrac{10,000}{\text{ohms}}\right)$ of a 1 per cent gelatin solution first rendered isoelectric and then treated with concentrations of an alkali, *e.g.* NaOH, varying from $M/8$ to $M/8192$ to cause a varying proportion of the gelatin to form a metal gelatinate. Abscissæ represent pH of the solution after dialysis; ordinates of upper curve, osmotic pressure, and of lower curve, conductivity of the solution found at different pH. Curves for the osmotic pressures for gelatin salts with univalent ion, Li, Na, K, and NH₄ (Figs. 2 to 5) are alike and high, reaching a maximum of about 325 mm. Curves for the osmotic pressures of salts with bivalent metals, Ca and Ba, are very much lower than those for metal gelatinates with univalent metal, reaching a maximum of only 125 mm. Curves for conductivity are almost identical for both types of gelatin salts.

once with 25 cc. of H$_2$O at 20°C. Then the mass was made into a 1 per cent solution and put into collodion bags to determine the osmotic pressure. Each collodion bag was surrounded by a beaker containing 400 cc. of distilled water and the temperature was kept constant at 24°C. This allowed any excess of alkali left to diffuse out of the bag. The next day the osmotic pressure was measured, the pH of the gela-
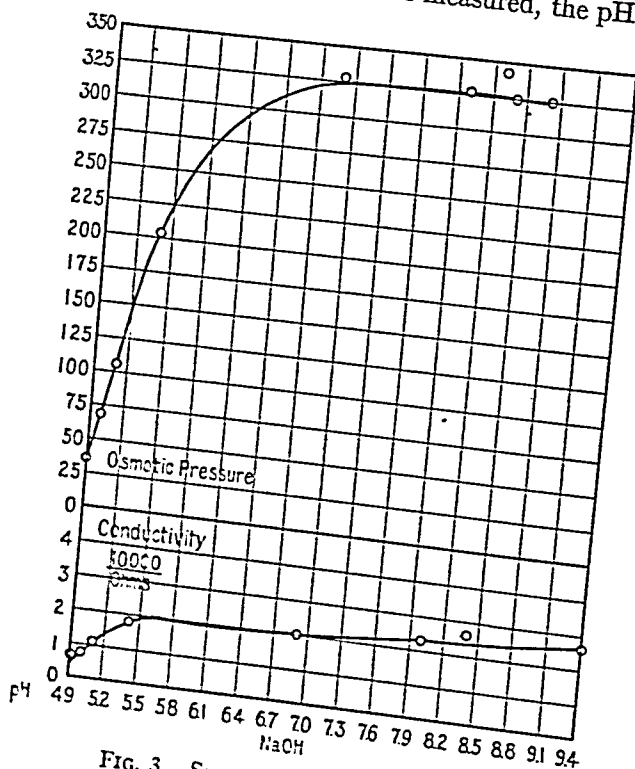

FIG. 3. See explanation under Fig. 2.

tin solution was determined, the solution titrated to determine the quantity of Na or K, etc., in combination with the gelatin, and the conductivity of the solution was measured.

The results of these measurements are contained in Figs. 2 to 7. The abscissæ are the pH, the ordinates the values for conductivity and osmotic pressure for these pH. The reader will notice that the curves representing the influence of Li, Na, K, and NH$_4$ on the osmotic

pressure (and the other physical properties) of gelatin are identical, if we use the pH as abscissæ.   This result contradicts the statements current in colloid chemistry according to which these four cations have a different effect.   The colloid chemists who make such statements have failed to measure the hydrogen ion concentration of their solutions.   Our experiments show that the effects of the same cation on
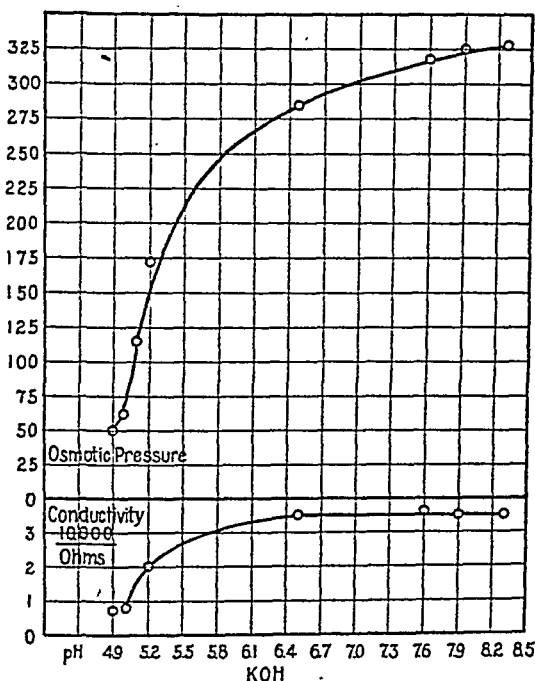

FIG. 4.   See explanation under Fig. 2.

gelatin differ for different pH and hence we cannot be sure that apparent differences in the effect of two cations on a protein are the expression of differences in the structure of the two cations, unless we are certain that the pH is the same in both cases.   The colloid chemists have, moreover, compared the effects of neutral salts of Li, Na, K, and NH₄, in the presence of an excess of these salts, which introduces a second error.

It is obvious that the osmotic pressure in the curves for LiOH, NaOH, KOH, and NH₄OH (Figs. 2 to 5) reaches the same maximum (at pH between 7.0 and 8.0), namely that of a column of about 325 mm. of a 1 per cent gelatin solution; and that the curves for osmotic pressure of the Ca gelatinate and Ba gelatinate (Figs. 6 and 7) reach also an equally definite maximum of about 125 mm. osmotic pressure at about the same pH. In both cases we have probably to deduct
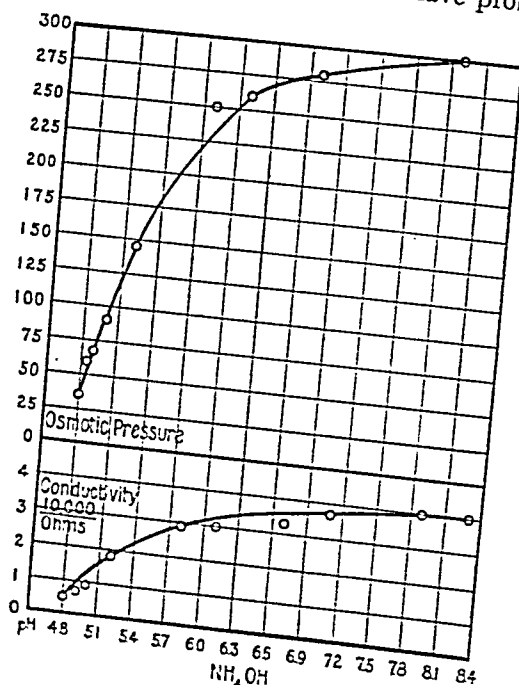

FIG. 5. See explanation under Fig. 2.

from these values about 25 mm.; namely, the osmotic pressure of a 1 per cent gelatin solution at the isoelectric point which includes the necessary capillary correction. This then leaves the following characteristic and constant values for the maximum osmotic pressure of the two types of metal gelatinates:

Li, Na, K, NH₄ gelatinate.......300 mm. (uncorrected value 325 mm.)
Ca and Ba gelatinate ....100 " (" " 125 ")

The constant character of this ratio of the two pressures of $1:3$ (or $3:8$ for the uncorrected value) for the two valencies, but regardless of the other qualities of the ions, betrays a stoichiometrical basis for the influence of valency.   The experiments were repeated to guard against error, the results remaining the same.
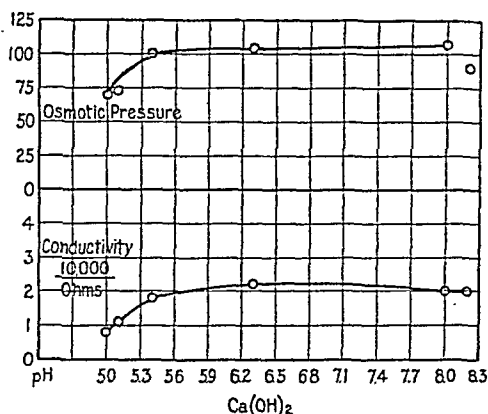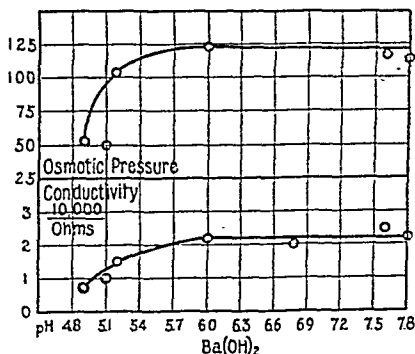

FIG. 6.   See explanation under Fig. 2.

FIG. 7.   See explanation under Fig. 2.

This means that in a 1 per cent solution of a metal gelatinate we have approximately three times as many particles in solution or suspension when the metal is univalent as when it is bivalent.   Before we can draw any further conclusions we have to consider the relative conductivities of the same gelatin solutions.

A glance at the curves for conductivity shows that those for Na, Li, Ba, and Ca are almost identical, while the curves for K and NH₄ are a little higher than the others. These experiments were repeated and the same values were obtained. Fig. 8 shows that while the curves for conductivity of gelatin salts with univalent and bivalent metals (Na and Ba) are almost identical, the curves for the osmotic pressure of the two types of salts are very different.
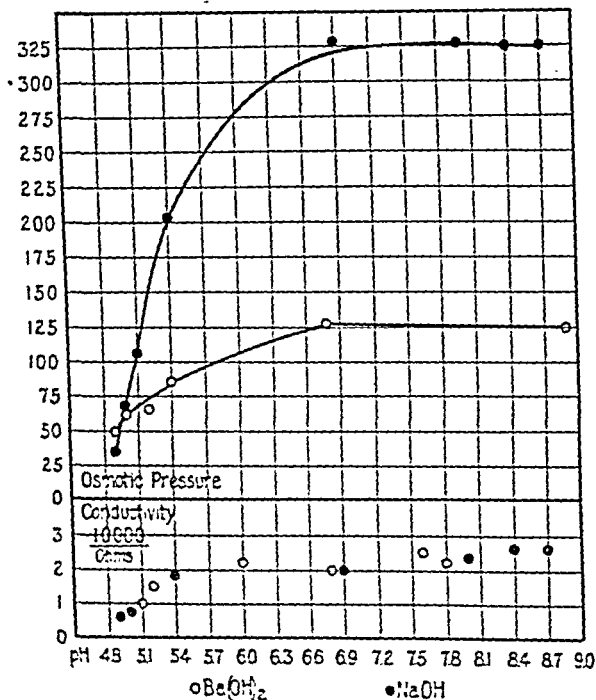

Fig. 8. Showing that while the curves for conductivity of sodium and barium gelatinate are practically identical, the curves for the osmotic pressures are very different.

There are two possible explanations for the fact that the ratio of conductivities of the two types of salts (Ca gelatinate and Na gelatinate) is 1:1 while the ratio of osmotic pressures is 1:3. The one explanation is that the degree of electrolytic dissociation of Ca gelatinate is so much smaller than that of Na gelatinate as to produce a

ratio of 1:3 particles in solution. This would account for the difference in the ratio of osmotic pressures but would leave unexplained the identity of conductivity of the two solutions. It would therefore be necessary to make a second assumption; namely, that the lower viscosity of a 1 per cent Ca gelatinate solution would raise the conductivity of a Ca gelatinate solution enough to compensate for the smaller degree of electrolytic dissociation.

In order to account for the ratio of 1:3 in osmotic pressure of the solutions of the two types of salts we have to assume that only about 20 to 25 per cent of the Ca gelatinate molecules are dissociated, while the dissociation of the sodium gelatinate is complete (five molecules of Ca gelatinate, one of which dissociates, would yield seven particles while the same amount of gelatin would form ten molecules of sodium gelatinate, yielding with complete dissociation twenty particles; this would result in a ratio of 7:20 for the relative number of particles in solution). The four electric charges of the one dissociated Ca gelatinate molecule would have to give the same conductivity as the twenty electric charges of the sodium gelatinate.

Our present knowledge speaks against such an influence of the viscosity of gelatin solutions upon conductivity. We prepared 1 per cent solutions of sodium, potassium, magnesium, and calcium gelatinate, of pH = 7.0, by putting 1 gm. of finely pulverized commercial Cooper's gelatin (probably mostly calcium gelatinate) for 1 hour at 20°C. into 100 cc. of M/4 NaCl or KCl, or MgCl₂ or CaCl₂, and allowed the excess of salt solution to drain off by putting the gelatin on a filter. We then washed the gelatin on each filter six times in succession with 25 cc. of H₂O, melted the gelatin by heating to about 50°C., and added enough water to make a 1 per cent gelatin solution. The solution was cooled to 24°C. and the time of outflow through a viscometer, as well as the conductivity of each solution, was measured immediately, at 24°C. (Table I). We found the usual typical difference in viscosity between Ca and Mg gelatinate on the one hand, and Na and K gelatinate on the other. It is well known that the viscosity of a gelatin solution prepared by melting will increase on standing, especially at a low temperature. The gelatin solutions were kept at about 2°C. for 2 hours and were then heated to 24°, and their viscosity and conductivity were again measured. All the

viscosities had increased considerably and the viscosity of magnesium and calcium gelatinate was now as great as was originally that of the sodium gelatinate. Yet the conductivities were practically unaltered. The experiment was continued as indicated in Table I and enormous viscosities resulted, practically without any increase in the conductivities.

The reader will notice incidentally from the continuation of the experiment that upon heating to 50°C. and cooling to 24° the viscosity went practically back to its original level for the four different gela-

TABLE I.

Variation of Viscosity and Conductivity of Gelatin Solutions upon Standing.

Viscosity in Seconds of Outflow, Conductivity, $\frac{10,000}{Ohms}$.

All Measurements at 24°C.

Treatment.	Na gelatinate.		K gelatinate.		Mg gelatinate.		Ca gelatinate.	
	Viscosity.	Conductivity.	Viscosity.	Conductivity.	Viscosity.	Conductivity.	Viscosity.	Conductivity.
	sec.	$\frac{10,000}{ohms}$	sec.	$\frac{10,000}{ohms}$	sec.	$\frac{10,000}{ohms}$	sec.	$\frac{10,000}{ohms}$
Immediately after melting...	99	2.31	102	3.07	88	2.10	86	2.12
After 1 hr. in refrigerator and heating to 24°C......	135	2.22	143	2.94	138	2.06	130	2.12
After 18 hrs. in refrigerator and heating to 24°C......	180	2.28	250	3.03	240	2.07	200	2.09
After being kept at 24° for 2 hrs................	170	2.35	210	3.09	194	2.10	173	2.13
After heating to 50° and cooling to 24°...........	94.5	2.35	95.5	3.11	86	2.12	83.5	2.14

tin salts. In this case the influence of the "history" upon the colloidal solution is entirely reversible.

These experiments seem to exclude the assumption that the degree of electrolytic dissociation of calcium gelatinate is so much smaller than that of sodium gelatinate that it produces a ratio of 1:3 in the osmotic pressure of the two solutions; and that the difference in the degree of dissociation is compensated by the influence of viscosity upon conductivity in such a way as to make the conductivities of the solution of the two types of salts equal.

The second possible explanation is based on the assumption that the equality of conductivity is due to the fact that both types of solutions in equal concentration and for the same pH possess an approximately equal number of charges.

The identity of equivalents combining with gelatin demands that twice as many gelatin molecules must combine with one atom of Ca as with one atom of Na. For the sake of simplification we assume that one Na atom combines with one gelatin atom. This would mean that calcium gelatinate exists in the form of Ca gelatin$_2$ or Ca$_2$ gelatin$_4$ or Ca$_3$ gelatin$_6$, and sodium gelatinate in the form of Na gelatin. In this case all phenomena will find their explanation if we assume that in the dissociation of Ca$_2$ gelatin$_4$ the four gelatin ions remain aggregated in one group with four negative charges

$$Ca_2 \, gelatin_4 \rightleftharpoons Ca^{++} + Ca^{++} + [gelatin]_4$$
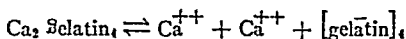
Such a dissociation would therefore yield three ions, one of which contains an aggregate of four negative gelatin ions. In order to obtain the same number of charges, four molecules of Na gelatin would be required, dissociating into four positive Na ions and four separate negative gelatin ions, making eight ions in all. This would demand a ratio of osmotic pressures for the two gelatin solutions of 3:8, which is slightly less than the ratio observed. The electrical charges would be the same for the two solutions and the conductivities would only show the difference due to differences in the ionic mobilities.

If the dissociating complex in the case of calcium gelatinate is Ca$_3$ gelatin$_6$ resulting in the formation of three Ca ions and one aggregate gelatin$_6$ anion carrying six charges, the same number of charges would be carried by six molecules of sodium gelatinate dissociating into twelve ions. This would yield exactly the ratio of 1:3 for the osmotic pressure of solutions of calcium gelatinate and sodium gelatinate of the same concentration and conductivity.

If the aggregates consist of eight gelatin anions with four Ca ions the ratio of osmotic pressures would be 5:16 which is also approximately 1:3.

It is in reality only necessary to assume the existence of compounds of the form Ca gelatin$_2$, the two anions of which form one aggregate of two gelatin anions, and to assume further that two, three,

more such aggregates of two gelatin anions join to form larger aggregates of four, six, or eight gelatin anions, every one of which k -+ its original charge. This would account for all the phenomena observed.

We are only able to estimate the relative difference in the mobilities of the cations. They are higher for K and NH₄ than for Na and Li, and we also find that the observed conductivities of K and NH₄ gelatinate in our experiments are higher than those of Na and Li gelatinate, possibly to the amount the difference in mobility of the ions named demands.

Bayliss in comparing the osmotic pressure and the electrolytic dissociation of solutions of Congo red found that it is ionized 80 per cent in a dilution of 500 liters, yet,

"The osmotic pressure [of such solutions] found experimentally, both by direct measurement and by vapour pressure, is, throughout a wide range of concentration, uniformly between 95 and 100 per cent. of what it would be if no dissociation existed. Since it should be from one and a half to three times this value, according to the concentration, it is plain that there are some abnormal conditions present."

Bayliss suggests an explanation similar to the one given above; namely, "the possibility of aggregated simple ions carrying the sum of the charges of their components."[8]

IV. Influence of Valency upon Swelling and V...

It has been demonstrated in previous papers th... ...e curves for the values of viscosity and swelling are similar to the curves for osmotic pressure and it has been stated that these properties must also be a function of the relative number of metal gelatinate molecules and ions formed.

In Figs. 9 and 10 we give the curves for viscosity and swelling of sodium and potassium gelatinate and in Figs. 11 and 12 the same curves for barium gelatinate and calcium gelatinate. The values for viscosity are given in times of outflow and the values for swelling in terms of the height of cyli...ers of gelatin of the same diameter.[9] On

[8] Bayliss, W. M., *Proc. Roy. Soc. London, Series B*, 1911, lxxxiv, 253, 254.
[9] Loeb, J., *J. Biol. Chem.*, 1918, xxxiv, 77, 395.